

1 **31° South: phenotypic flexibility in adaptive thermogenesis among conspecific populations**
2 **of an arid-endemic bird - from organismal to cellular level**

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15 lipids

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17

18 **ABSTRACT**

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

33

34 INTRODUCTION

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

41 At cold temperatures, homeothermic-endotherm animals (i.e.: birds and mammals) loose heat
42 from their warm bodies to the environment. To maintain a high body temperature in cold
43 conditions, birds principally increase the rate of heat production through shivering (Hothola
44 2004). While shivering thermogenesis results from cellular processes, namely the activation of
45 energetic metabolism pathways to burn cellular fuels to power skeletal muscle contraction
46 (Hothola 2004), it can be quantified at the organism-level as the maximum thermoregulatory
47 metabolic capacity (M_{sum} ; (Thompson 2010)). In fact, several studies showed that cold
48 temperatures lead to increases in M_{sum} (Vezina *et al.* 2011) (Swanson *et al.* 2014a). One
49 explanation for this flexibility in maximum metabolic capacity is the cold adaptation hypothesis,
50 which posits that birds wintering in cold climates should have higher M_{sum} than those in warmer
51 climates, and hence high M_{sum} is critical for overwinter survival in very cold regions (Marsh and
52 Dawson 1989) (Swanson and Garland 2009). An alternative explanation is offered by the climate
53 variability hypothesis (Janzen 1967), (Bozinovic and Naya 2014), which posits that broader
54 climatic fluctuations results in wider flexibility in thermal tolerance as a means to cope with the
55 fluctuating environmental conditions.

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

58 mass (Vézina *et al.* 2006) (Zheng *et al.* 2014); ii) increase in muscle mass, in particular the
59 pectoralis muscle which is the primary thermogenic organ (Vézina *et al.* 2006) (Swanson *et al.*
60 2014a); iii) increase in enzymatic activity in oxidative metabolic pathways (Vézina *et al.* 2006)
61 (Zheng *et al.* 2014) (Liknes and Swanson 2011a); and iv) increase in fat catabolism (Dawson *et*
62 *al.* 1992) (Thompson 2010). However, evidence for flexibility in M_{sum} , as well as support for the
63 above mentioned mechanisms driving up-regulation of M_{sum} , comes primarily from endotherms
64 living in north-temperate ecosystems, where summer primary productivity is high (Prince and
65 Goward 1995), and therefore individuals can afford the large energetic cost of up-regulating
66 metabolic heat production (Hothola 2004).

67 In ecosystems characterized by low primary productivity and large seasonal temperature
68 fluctuations, it is not clear how endotherms survive the low winter temperatures if not using
69 energy saving strategies such as heterothermy. Therefore, to improve our understanding of the
70 physiological underpinnings of adaptive thermogenesis in such ecosystems, we studied a small
71 passerine (Karoo scrub-robin, *Cercotrichas coryphaeus*; hereafter designated scrub-robin) living
72 in the subtropical arid-zone of southwestern Africa. Its range spans over an area of overall low
73 primary productivity (Prince and Goward 1995) and exhibits a thermal gradient from west to east
74 (Figure1C). As one transects from the Atlantic coast to inland, winters become increasingly
75 colder, and primary productivity decreases as rainfall becomes increasingly unpredictable.
76 Specifically, along the Atlantic coast winters are mild (mean minima: 8°C, record minima >0°C)
77 and moderately productive from regular fog and predictable winter rainfall; in contrast, in the
78 continental region minimum temperatures reach sub-zero (mean minima: -3°C, record minima <-
79 10°C) and primary productivity is very low year-round. Given these environmental features, the
80 scrub-robin renders an ideal system for testing whether sub-tropical birds fine-tune phenotypes
81 associated with adaptive thermogenesis in response to local environmental conditions. We
82 hypothesize that birds from populations experiencing sub-zero winter temperatures would

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

95

96 **METHODOLOGY**

97 ***Study sites, sampling and ethical clearance***

98 We selected study sites that represent three main regions along the climatic and primary
99 productivity gradient in the scrub-robin range (Figure 1C): *Coastal*, *Central* and *Inland*. We
100 captured 85 adult birds in three regions and two seasons (summer: December 2015; winter: July
101 2016): $n_{\text{coastal}} = 32$ ($n_{\text{summer}} = 18$; $n_{\text{winter}} = 14$), $n_{\text{central}} = 27$ ($n_{\text{summer}} = 14$; $n_{\text{winter}} = 13$) and $n_{\text{inland}} = 26$
102 ($n_{\text{summer}} = 10$, $n_{\text{winter}} = 16$). Birds were captured using spring traps baited with mealworms
103 (*Tenebrio molitor*). We kept them in individual cages in a quiet room with *ad libitum* access to
104 food for no more than 24h, when the metabolic experiments took place. We released the birds at
105 the point of capture, except those sacrificed (details below). Each sampling location was
106 georeferenced and the GPS coordinates were the used to extract mean minimum temperature
107 (T_{min}) and Normalised Difference Vegetation Index (NDVI), a surrogate for primary productivity,

108 for the months of data collection. T_{\min} was extracted from two sources: i) WorldClim V2
109 database (<http://worldclim.org/version2>; (Fick and Hijmans 2017)) which is averaged from 1970-
110 2000 interpolated at 2.5min resolution (~5km) and ii) South African Weather Service for three
111 weather stations closest to our study and contemporary to our experiments. NDVI at 250m
112 resolution from USGS-LandDAAC-MODIS dataset hosted by United States Geological Survey
113 (https://lpdaac.usgs.gov/dataset_discovery/modis).

114 Permits to capture, handle and sacrifice birds were issued by the Northern Cape Department of
115 Environmental Affairs (ODB 2665 & 2666/2015; Northern Cape Province) and CapeNature
116 (0056-AAA008-00051; Western Cape Province) in South Africa. The Animal Ethical Committee
117 at Nelson Mandela Metropolitan University (South Africa) approved all experiments (A15-SCI-
118 ZOO-005).

119

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

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

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

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

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

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

141

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

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

157 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 $\sim 1.5 \text{ L min}^{-1}$ 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 $\sim 1.5 \text{ L min}^{-1}$) to allow the bird to calm down. Trials started at air temperature ranging 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) $T_b < 34 \text{ }^\circ\text{C}$. Unlike previously published studies on
199 passerines undergoing cold exposure that were terminated when birds reached $T_b < 37 \text{ }^\circ\text{C}$
200 (Swanson et al. 1996) we found that many scrub-robins showed a decline in T_b well below $37 \text{ }^\circ\text{C}$,
201 while still increasing resting metabolism and hence the reason for establishing the $T_b < 34 \text{ }^\circ\text{C}$
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

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

214

215 ***Statistics***

216 We extracted NDVI and T_{min} values for our sampling sites using the R *Raster* package (Hijmans
217 2017). For all variables, we tested for normality and homogeneity of variance using Shapiro–
218 Wilk’s and Levene’s test, respectively. If heteroscedasticity was detected, the response variable
219 was log-transformed. We used an analysis of variance to test for sexual dimorphism. In the event
220 that sex was not significant, we removed the variable and proceeded with pooled sexes. We tested
221 for the role of environmental features such as T_{min} and NDVI on body condition, mass of
222 thermogenic muscles (mass of pectoralis and gastrocnemius) and cellular aerobic capacity
223 (density of mitochondria and lipid droplets), cold limit (T_{CL}) and thermal conductance using
224 generalised linear models (GLMs). We opted to use T_{min} experienced during each study period as
225 a linear predictor instead of “season” as a categorical predictor as we believe that variation
226 amongst study site in the former metric would explain physiological responses better.

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

229 All statistical analyses were performed in R v3.3.2 (R Development Core Team) and plots
230 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 ($M_{b-scaled}$). Birds significantly increased body
241 condition as T_{\min} decreased (GLM; $t_{T_{\min}} = -3.666$, $p < 0.01$); FIGURE 2A-B). At the regional
242 level, the decrease of T_{\min} and NDVI lead to a significant increase of $M_{b-scaled}$ for birds living in
243 the *Inland* (GLM, $t_{T_{\min}} = 2.254$ $p=0.03$; $t_{NDVI} = 3.229$, $p < 0.01$) and *Coastal* (GLM, $t_{T_{\min}} = -2.763$,
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, $t_{T_{\min}}$
247 $= -14.795$, $p < 0.01$; FIGURE 2C-D). The amount of visible fat significantly increased as T_{\min}
248 decreased in *Coastal* (GLM, $t_{T_{\min}} = -4.295$, $p < 0.01$), *Central* (GLM, $t_{T_{\min}} = -6.245$, $p < 0.01$) and
249 *Inland* (GLM, $t_{T_{\min}} = -2.421$, $p = 0.02$) 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 \pm SE = $1.114 \pm 0.034g \times 2 = 2.228 \pm 0.068$ g)
254 accounted for 12.3% of scrub-robin body mass ($n = 15$, mean \pm SE: $18.8g \pm 1.382$ g), while the
255 gastrocnemius ($n = 15$, mean \pm SE: 0.089 ± 0.002 g $\times 2 = 0.178 \pm 0.004$ 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***

280 We observed no sexual dimorphism in maximum thermoregulatory metabolic capacity (M_{sum} ;
281 ANOVA, $F = 0.202$, $p > 0.1$). Maximum thermogenic capacity (M_{sum}) ranged from 1.373 to 3.309
282 mL CO_2/min , with mean = $2.341 \text{ mL CO}_2/\text{min} \pm 0.384 \text{ SD}$. M_{sum} was significantly positively
283 related with T_{min} (GLM, $t = 2.764$, $p < 0.01$) and $M_{\text{b-scaled}}$ (GLM, $t = 2.703$, $p < 0.01$) but not with
284 NDVI (GLM, $t = 1.044$, $p > 0.1$).

285 When restricting the analysis to regional-level, we found that M_{sum} increased alongside with
286 increasing T_{min} (i.e. summer) in *Coastal* (GLM, $t = 2.903$, $p = 0.01$) and *Central* (GLM, $t =$
287 2.577 , $p = 0.02$) populations alongside increasing T_{min} (i.e. summer), whereas birds from *Inland*
288 showed no significant changes (GLM, $t = 1.446$, $p = 0.163$; Figure 2E).

289 At maximum thermogenic capacity, scrub-robins defended similar body temperatures regardless
290 of local T_{min} (GLM; $t = 1.510$, $p > 0.1$). Nevertheless, the temperature that triggered the
291 maximum heat production - cold limit (T_{cL}) - decreased at lower T_{min} (GLM, $t = 7.661$, $p < 0.01$;
292 Figure 2F). This association that was significant for the three regions: *Coastal* (GLM, $t = 2.523$, p
293 $= 0.019$), *Central* (GLM, $t = 2.634$, $p = 0.017$) and *Inland* (GLM, $t = 5.034$, $p < 0.01$).

294 Overall, the rate of metabolic heat loss was positively associated with increasing T_{min} (GLM; $t =$
295 4.858 , $p < 0.01$) but not with $M_{\text{b-scaled}}$ (GLM; $t = 1.471$, $p > 0.1$). Within-region, thermal
296 conductance significantly increased with increasing T_{min} in *Coastal* and *Central* populations
297 (GLM; $t_{\text{Coastal}} = 3.966$, $p < 0.01$; $t_{\text{Central}} = 4.976$, $p < 0.01$), while no association with T_{min} was
298 found for *Inland* birds (GLM; $t_{\text{Inland}} = 1.962$, $p = 0.064$).

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

304

305 **DISCUSSION**

306 Cold temperatures have long been recognized as an inescapable physiological stressor for small-
307 bodied endotherms such as birds (Tattersall *et al.* 2012). While small birds from north-temperate
308 habitats deal with this challenge through flexibility in thermoregulatory physiological traits (e.g.:
309 (Dawson and Olson 2003), (Vézina *et al.* 2006)), there is a paucity of information on whether
310 sub-tropical species use similar mechanisms (Smit and McKechnie 2010), as well as whether
311 intra-specific variation allows populations to acclimatize to local conditions (van de Ven *et al.*
312 2013).

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

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

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

333

334 ***Thermogenesis in sub-tropical arid-zones***

335 Although M_{sum} revealed to be seasonally and regionally flexible, the observed patterns contrast
336 with our predictions. Firstly, M_{sum} flexibility was only found in populations living in the milder
337 portion of the scrub-robin range (*Coastal* and *Central*) and not in the population exposed to sub-
338 zero T_{min} in winter (*Inland*). And secondly, the trend was to increase thermogenic capacity in
339 summer, the period where M_{sum} was expected to be at an annual minimum (Swanson and Garland
340 2009) due to annual highest T_{min} . Our estimates of M_{sum} (assuming an respiratory exchange ratio
341 of 0.71) are, on average, 75% of the values predicted by Swanson and Bozinovic (2011) for a 19
342 g oscines passerine, suggesting that M_{sum} is not directly linked to cold tolerance in this arid-zone
343 passerine.

344 Our findings contrast with cold-induced increased of M_{sum} in north-temperate passerines (e.g.:
345 American Goldfinch *Spinus tristis* (Swanson et al. 2014b), House sparrow *Passer domesticus*
346 (Liknes and Swanson 2011a) and are only comparable to results reported for two other southern
347 African birds living in similar latitudes in South Africa: the red-bishop (*Euplectes orix*; (van de
348 Ven et al. 2013) and the white-browed sparrow-weaver (*Plocepasser mahali*; (Noakes et al.
349 2017). Both studies found population-specific patterns in M_{sum} flexibility, with some populations
350 increasing M_{sum} in winter (in colder sites), while others showed no seasonal change.

351 Although the lack of increased M_{sum} in scrub-robins at colder temperatures was unexpected, it
352 was consistent with the unchanged mass of the major thermogenic muscles (pectoralis and
353 gastrocnemius). In contrast to northern-temperate Passeriformes that increase the mass of
354 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 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 $T_{\text{min}} = 13$ °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 ($T_{\text{min}} = -0.4$ °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.

379

380 *Thermosensation prior to thermogenesis*

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

388 Increasing locomotor activity exploits the thermodynamic inefficiency of catabolic reactions in
389 skeletal muscles (60% energy released as heat) and nonshivering thermogenesis uses a deflection
390 of proton flux in mitochondria from ATP production to heat production (Hochachka and Somero
391 2002). However, none of the mechanisms seem plausible in the scrub-robin. First, because being
392 on the move for the sake of heat production in low productivity environments may lead to a
393 mismatch between energetic demand and supply. And secondly, recent work suggests that
394 nonshivering thermogenesis is not a prominent contributor to thermogenesis in adult passerines
395 (Cheviron and Swanson 2017). With neither locomotor activity nor nonshivering thermogenesis
396 responsible for thermoregulation, we propose the explanation may be on how the scrub-robins
397 sense the cold. This idea is supported by the fact we recorded a drastic reduction in T_b to 34 – 36
398 °C, while birds maintained their maximum metabolic capacity, and remarkably that scrub-robins
399 could fly at ease at $T_b = 30 - 34$ °C, soon after the cold-experiment. It is thus possible that intra-
400 specific variation exists in the somatosensory system (cutaneous thermoreceptors; e.g.: TPRM8
401 channel (Matos-Cruz *et al.* 2017)) and hence some populations tolerated prolonged exposure to
402 cold and hypothermia without engaging in active production of heat. An understanding of the
403 sensory perception of cold in Karoo scrub-robin, as the first system to mediate organism-
404 environment interactions (Gracheva and Bagriantsev 2015), is certainly warranted.

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

408

409

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417

418 **Author contributions**

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

423

424 **Data accessibility**

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

426

427 **LITERATURE CITED**

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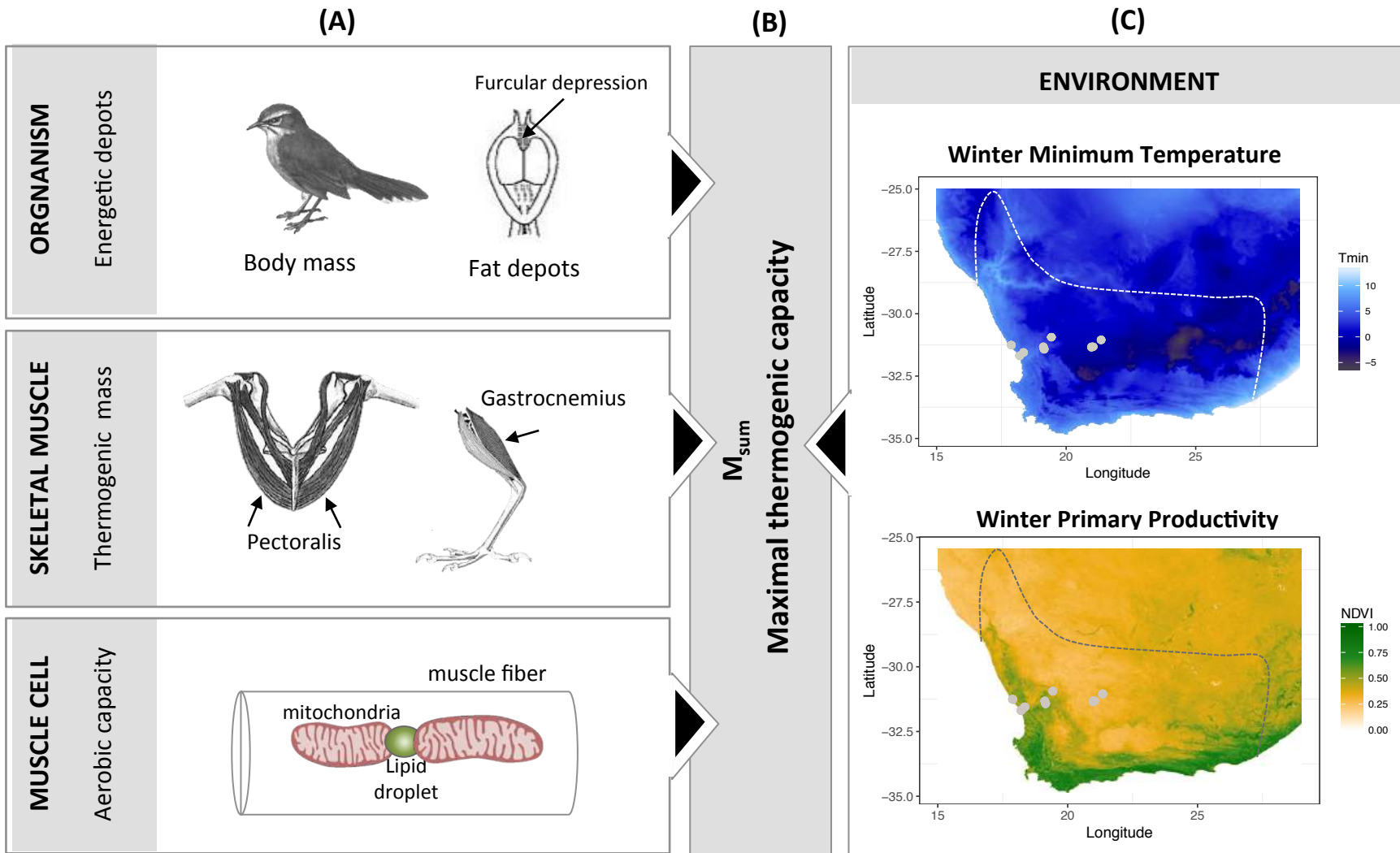


Figure 1. Framework to study the mechanisms of adaptive thermogenesis in small birds living in arid-zones. (A) The underlying mechanisms driving (B) flexibility in maximal thermogenic capacity, and the role of (C) environmental variables in shaping those mechanisms. Study sites relative to the species range (delimited by a dashed line) are depicted in the maps.

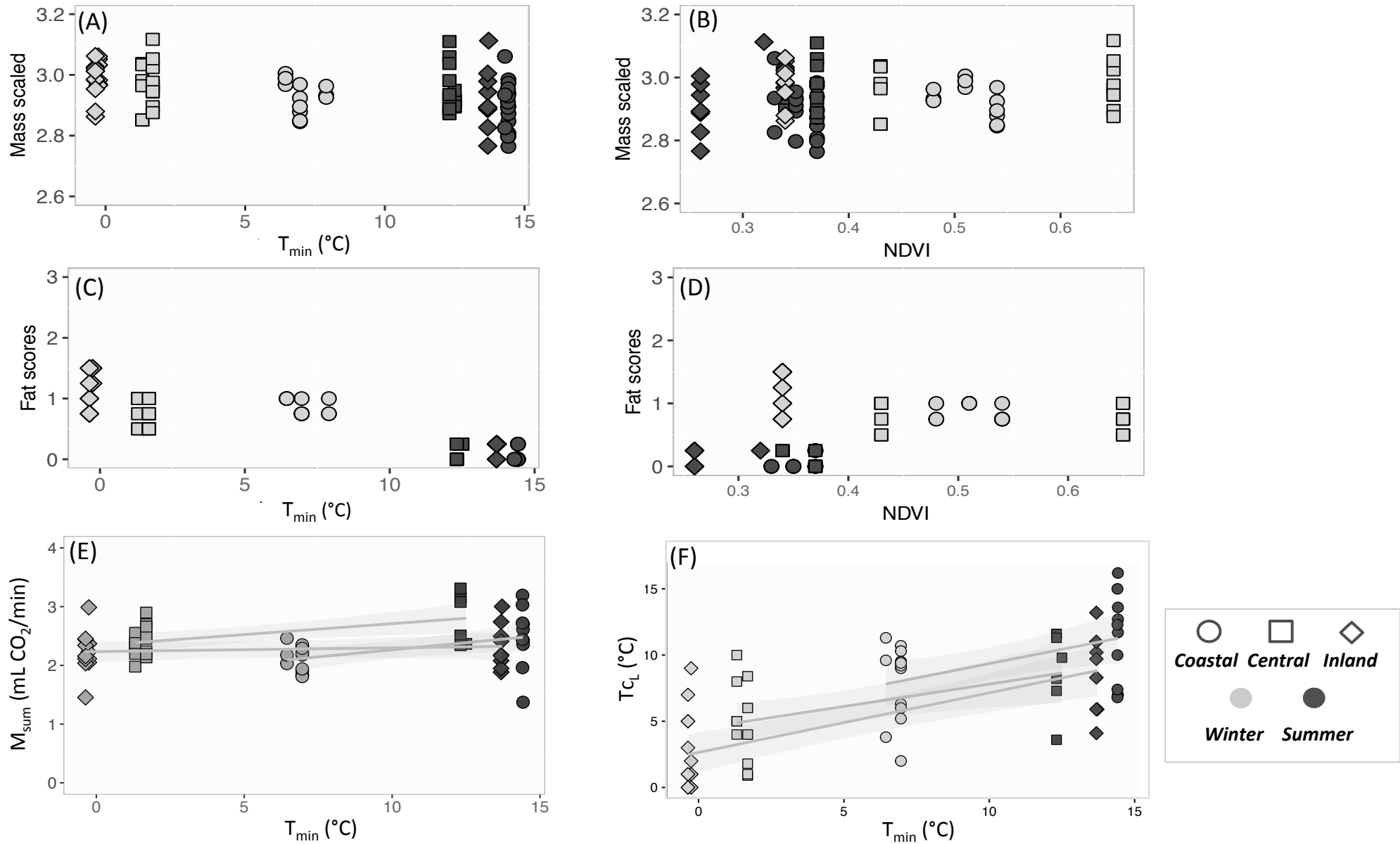


Figure 2. Variation in physiological traits in response to mean minimum temperature (T_{\min}) and primary productivity (NDVI). (A-B) Mass scaled, (C-D) Fat scores, (E) maximum thermogenic metabolic capacity (M_{sum}) and (F) cold limit (T_{CL}).

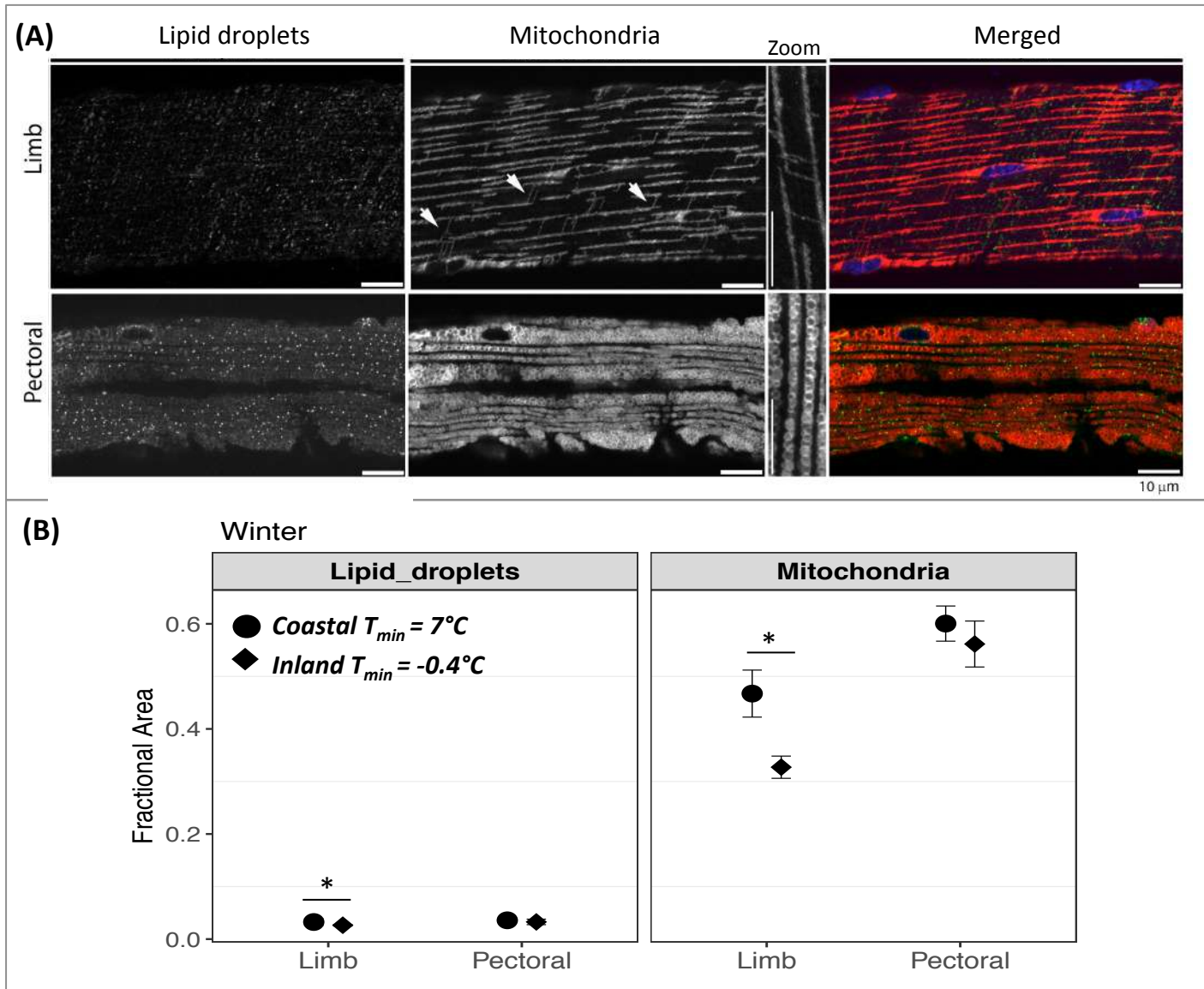


Figure 3. Variation in aerobic capacity in thermogenic muscles. (A) Confocal micrographs depicting mitochondria and lipid droplets (Perilipin2). Merged image show mitochondria (red), lipid droplets (green) and nuclei (blue). Transversal connections between mitochondria, exclusive of limb fibers, are indicated with arrows and visualized in zooming in images. Bars: 10 μ m. (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.