Metabolic enzymes in seasonally acclimatized and cold acclimated rufous-collared sparrow inhabiting a Chilean Mediterranean environment

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Abstract Due to the higher energy requirements of birds during winter, it is predicted that the activities of metabolic enzymes (e.g., citrate synthase, CS and cytochrome C oxidase, COX) should increase in tandem with increases in rates of energy expenditure (e.g., basal metabolic rate, BMR). However, there is mixed support for the hypothesis of enzymatic acclimatization. Furthermore, there is little information about the effect of ambient temperature on energetics and tissue enzyme activity levels in passerines inhabiting seasonal Mediterranean environments. In this study we evaluated the interplay between BMR and enzyme activities of freshly caught individuals of the passerine Zonotrichia capensis in winter and summer in a Mediterranean environment from central Chile, and also in warm (30°C) and cold (15°C) lab-acclimated birds. The results revealed a lack of seasonal variation in BMR, thermal conductance and in the activity of CS and COX. However, we found higher BMR and lower thermal conductance in cold-acclimated than in warm-acclimated birds. Also, total CS activity was higher in the flight muscles of cold-acclimated than in warm-acclimated birds. We found also a significant correlation between BMR and total CS activity in pectoral muscle. Although some authors have suggested that BMR mainly depends on the metabolic intensity and mass of internal organs, our results revealed that skeletal muscles such as the flight muscles may also affect BMR. Finally, differences in the acclimation and acclimatization responses in Rufous-collared sparrows suggest that large-scale oscillations in the physical environment might maintain the capacity for flexibility in thermogenic traits through generations [Current Zoology 60 (3): 338–350, 2014].

Keywords Basal metabolic rate, Citrate synthase, Cytochrome C oxidase, Phenotypic flexibility, Thermal acclimation, Zonotrichia capensis

Animals expend energy in different seasons at variable rates, depending on changes in environmental abiotic and biotic conditions and the resulting physiological demand (Bozinovic et al., 1990, 2004; Bozinovic and Merritt, 1991). Most research attention involving seasonal variation in physiological traits in birds has been paid to metabolic variation between summer and winter in strongly seasonal environments (high altitude and latitudes, e.g. Cooper and Swanson, 1994; Swanson, 2010; Smit and McKechnie, 2010), where climate is characterized by large seasonal temperature differences, with warm to hot and frequently humid summers and very cold and snowy winters. Surprisingly little effort has been directed toward testing hypotheses of seasonal changes in physiological traits in birds inhabiting Mediterranean environments (but see López-Calleja and Bozinovic, 2003; Maldonado et al., 2009). The Mediterranean-type climate is transitional between tropical arid and humid temperate. Despite being seasonal, those regions with Mediterranean climates have relatively mild winters but warm summers; nevertheless, seasonality in ambient temperatures can vary greatly between different regions with a Mediterranean climate.

Because of climatic seasonality, bird species that do not migrate must often cope with fluctuating environmental conditions such as changes in environmental temperature, photoperiod, water and food availability (Maddocks and Geiser, 2000). In Mediterranean zones at higher latitudes during winter, climatic conditions and productivity deteriorate. Also, climatic seasonality as well as existing or future extreme climatic events could be an important selective force acting on organisms in the wild. Thus, resident species in Mediterranean zones must cope with the effects of short days, long...
nights of forced fasting, relatively low food availability and cold temperatures (Jahn et al., 2010; Swanson, 2010). These species are expected to exhibit reversible (seasonal) phenotypic variation in their physiology (Dawson et al., 1983; Tieleman et al., 2002; Piersma and Vézina, 2007) as a response to their variable environments (Kingsolver and Huey, 1998). This type of response seems to allow organisms to respond to environmental variation at shorter than evolutionary time scales (de Jong, 1995; Futuyma and Moreno, 1988; Bicudo et al., 2010; Piersma and van Gils, 2011). Nevertheless, the plastic response, if present (see Maldonado et al., 2009), may also be diverse, depending on the magnitude of climatic seasonality and its variability between different regions and climates (Smit and McKechnie, 2010).

Short-term changes (seasonal acclimatization and laboratory acclimation) in basal metabolic rate (BMR) have been documented in several bird species (e.g., Swanson, 1991a, b; Cooper and Swanson, 1994; Piersma et al., 1995; Williams and Tieleman, 2000; Cooper 2002; Tieleman et al., 2003). In general, studies documenting thermal acclimation under controlled conditions in the laboratory found elevated BMR in birds at cold temperatures and a decrease in BMR when individuals were maintained in warmer environments (e.g., Klaassen et al., 2004; McKechnie et al., 2007; Maldonado et al., 2009; Barceló et al., 2009). Short-term changes in BMR are thought to arise, among other factors, from adjustments in the mass of metabolically active organs and the specific metabolic activity of these organs (Daan et al., 1990, Weber and Piersma, 1996; Piersma and Lindström, 1997; Kersten et al., 1998; Piersma et al., 1999; Zheng et al., 2008). Some studies have evaluated the presence of seasonal plasticity of the activity of enzymes indicative of tissue aerobic capacity in passerines, such as citrate synthase (CS, regulatory enzyme that catalyzes the first step of the tricarboxylic acid cycle) and cytochrome c oxidase (COX, an enzyme in the electron transport chain that catalyzes the reduction of oxygen to water; see Hochachka et al., 1988, Ludwig et al., 2001). Due to the usual increases in BMR of birds during winter or at low ambient temperature, it is predicted that the activities of these enzymes should increase in tandem with increases in rates of energy expenditure (Vézina et al., 2006; Zheng et al., 2008; Liknes and Swanson, 2011). However, there is mixed support for the hypothesis of enzymatic acclimatization, because it depends upon the species studied, the tissue type (e.g. muscles or liver) and the enzymes studied (Marsh and Dawson, 1982; Yacoe and Dawson 1983, Carey et al., 1989; O’Connor, 1995; Liknes, 2005; Zheng et al., 2008; Swanson, 2010; Liknes and Swanson, 2011; Zheng et al., 2013). As mentioned above, there is little information about the effect of temperature on enzyme activity levels in passerines inhabiting Mediterranean environments and to the best of our knowledge no studies has evaluated simultaneously the thermal acclimation and seasonal acclimatization of BMR and metabolic enzymes in the same species. The lack of such kind of studies prevents an accurate determination of the relative importance of the factors affecting rates of metabolic enzyme activity in birds (see Saarela and Hohtola, 2003). In this study we evaluated the interplay between BMR and enzyme activities of freshly caught individuals of a passerine species -- the rufous-collared sparrow Zonotrichia capensis -- in winter and summer in a Mediterranean environment from central Chile, and also in warm- and cold-acclimated sedentary individuals in the laboratory. The climate in central Chile is of temperate Mediterranean type, with 65% of the annual mean precipitation concentrated in winter from June to August. Precipitation is minimal from December to March, with only 3% of the yearly total. Temperatures are highest from December to March, corresponding to the austral summer, and lowest from June to August, the austral winter. On average, the mean minimum temperature is about 6°C below the mean temperature (http://www.meteochile.gob.cl/).

1 Material and Methods

1.1 Field methods and study site

1.1.1 Acclimatization

A total of 10 adult birds were captured by mist net in February 2012 (austral summer); 10 were captured in July 2012 (austral winter) in the Quebrada de la Plata, Chile (33°28′S, 70°54′W). Following capture, we transported them 30 km to a laboratory in Santiago, Chile (33°27′S, 70°42′W). Here birds were held in individual plastic-mesh cages (35 × 35 × 35 cm) in a constant temperature room (22 ± 2°C) on a 12L:12D photoperiod. While in captivity the sparrows consumed mealworms, bird seed and water, which were available ad libitum. After habituating birds to laboratory conditions for 1 d, we measured BMR (mL O₂ h⁻¹) at 30°C, which is within the thermoneutral zone for this species (Sabat et al., 2006). Average temperature data from a meteorological station, about 10 km from the study site, indicated that the mean maximum temperature of the winter (July) and summer (January) periods were ca. 15.8 ± 5.1 °C and
28.1 ± 2.9 °C, whereas mean minimum temperatures were ca. 5.5 ± 3.0 °C and 11.3 ± 2.0 °C for winter and summer, respectively (Pudahuel meteorological station, Dirección Meteorológica de Chile, http://www.meteochile.gob.cl).

1.1.2 Laboratory Acclimation

Nineteen birds were captured in May, 2012. In the laboratory, birds were housed individually in plastic-mesh cages (35 × 35 × 35 cm) under a 12L:12D photoperiod at 22 ± 1 °C for two days. Following capture, each bird was assigned to one of two treatments: one group was housed in a constant temperature room at 15 ± 1 °C (cold-acclimated group, n = 9) and the other at 30 ± 1 °C (warm-acclimated group, n = 10). These two temperatures are comparable to the mean of daily maximum temperatures experienced by birds in the field in winter and summer, respectively. We were confident that these two temperatures represent contrasting thermoregulatory demands because in a previous study we observed significant differences in BMR between cold and warm acclimated birds (Maldonado et al., 2009). After an acclimation period of six weeks, we measured BMR in both the cold- and warm-acclimated birds at 30°C.

1.2 Basal metabolic rate

Measurements of BMR were made in post-absorptive (4-h fasted), resting birds during the inactive phase and within the thermoneutral zone of the species, using standard flow-through respirometry methods. Briefly, birds were weighed, placed in a dark metabolic chamber (2 L), and then placed in a controlled temperature cabinet (Sable Systems, Henderson, Nevada) at a constant ambient temperature (T_a = 30 ± 0.5 °C). The metabolic chamber received dried air at 500 mL min^{-1} from a mass flow controller and through Bev-A-Line tubing (Thermoplastic Processes Inc.). The excurrent air passed through columns of Drierite, CO2-absorbent granules of Baralyme and Drierite before passing through an O2-analyzer, Turbo Fox model (Sable Systems, Henderson, Nevada) calibrated with a known mix of oxygen (20%) and nitrogen (80%) that was certified by chromatography (INDURA, Chile). The mass flow meter of the Turbo Fox was calibrated monthly with a volumetric (bubble) flow meter. The measurement and calibration protocols followed those of Tieleman and Williams (2000). Because water vapor and CO2 were scrubbed before entering the O2 analyzer, oxygen consumption was calculated as [Withers (1977: p 122)]: VO2 = \[(FR \times 60 \times (F_i \text{O}_2 - F_e \text{O}_2)) / (1 - F_e \text{O}_2)\], where FR is the flow rate in ml/min after STP correction, and F_i and F_e are the fractional concentrations of O2 entering and leaving the metabolic chamber, respectively. Body mass was measured before the metabolic measurements using an electronic balance (± 0.1 g) and cloacal body temperature (T_b) was recorded at the end of each measurement with a thin Cole- Palmer copper-constantan thermocouple attached to a Digisense thermometer (Model 92800-15). We found that all animals were euthermic after the metabolic trials (T_b > 40°C). Output from the oxygen analyzer (%) and flow meter were digitalized using a Universal Interface II (Sable Systems) and recorded on a personal computer using EXPEDATA data acquisition software (Sable Systems). Our sampling interval was 5 s. Birds remained in the chamber overnight to achieve steady-state conditions. This typically occurs after 6–8 hours for small birds (Page et al., 2011) and for Z. capensis in particular (Sabat et al., 2010). Thermal conductance (C) was calculated by C = VO2/ (T_b – T_a) by measuring VO2 at 15°C. We were confident that such temperature present birds with significant thermoregulatory demands because the rise in oxygen consumption over the BMR at 15°C is near 64% in Z. capensis (Sabat et al., 2006). We averaged O2 concentration of the excurrent air stream over a 20-min period after steady-state was reached (following Tieleman et al., 2002). We report whole-organism metabolic rates for seasonal and acclimation comparisons to avoid the potentially confounding problems of different body composition between seasons or acclimation treatments (see Swanson 1991a). In addition, with values of C, BMR and T_b, we estimated the lower critical temperature (Tlc) as Tlc = T_b – BMR/C. In all cases Tlc was < 30°C (25.7 ± 2.9 °C; 24.6 ± 3.1 °C; 22.4 ± 2.1 °C; 24.9 ± 3.1 °C for winter, summer, cold-acclimated and warm-acclimated birds respectively), confirming that our BMR measurements were performed within the thermoneutral zone. After metabolic determinations, animals were sacrificed by CO2 exposure, weighed and dissected to remove the internal organs. To the best of our knowledge, no studies has estimated the effect of euthanasia by CO2 exposure on CS and COX activities. However, any potential reduction in activity due to this method of euthanasia would affect all animals in the same way, allowing reliable comparisons among groups. Organs (liver, small intestine, kidneys and heart) and muscles (flight muscles and legs) were removed from the birds, weighed (±0.0001 g) and the liver and muscles were frozen at -80°C for further enzymatic determinations.

1.3 Enzyme determinations

Enzymatic activity determinations were performed in
homogenates of muscle and liver tissues. To do this, immediately after sacrifice, animals were dissected on ice and the liver, pectoralis and supracoracoideus were extracted, along with the major muscles of both lower limbs (iliotibialis cranialis, iliotibialis lateralis, iliofibularis, femorotibialis externus, fibularis longus, tibialis cranialis, extensor digitorum longus and gastrocnemius complex) (Liknes and Swanson, 2011). For the analyses we used the homogenates of the entire flight muscles (pectoralis plus supracoracoideus), the set of muscles of the lower extremities and the liver. The tissues were stored at -80 °C for further enzyme assays. Because we did not obtain limb muscles from the animals in winter, we only report the activity of flight muscles and liver in the seasonal section of the study.

Tissues were thawed, weighed and homogenized in 10 volumes of phosphate buffer 0.1 M with EDTA 0.002 M (pH 7.3) with an Ultra Turrax homogenizer (20000 rpm) on ice to avoid enzymatic reactions. Samples were then sonicated at 130 watt for 20 seconds with 10 seconds intervals, 14 times each, using an Ultrasonic Processor VCX 130, maintaining the samples on ice. Cellular debris was removed by centrifugation for 15 min at 15,000 rpm and 4°C. The supernatant was carefully transferred into a new tube, avoiding co-transport of the upper lipid layer present in the liver preparations. Protein concentration of the samples was determined using the method described by Bradford (1976), using bovine serum albumin as standard. Activities of two mitochondrial enzymes were determined: cytochrome c oxidase (COX; E.C. 1.9.3.1), and citrate synthase (CS; E.C. 4.1.3.7). Increments in these parameters likely reflect modifications in both the properties and the numbers of the mitochondria present (Guderley, 1998). COX activities were determined spectrophotometrically according to Moyes et al. (1997), with slight modifications. Enzyme activity was determined in 10mM Tris/HCl pH 7 containing 120 mM KCl, 250mM sucrose, and cytochrome c reduced with dithiothreitol to a final volume of 0.2 ml. The decrease in D.O. at 550nm was monitored in a thermo Scientific Multiskan GO spectrophotometer at 25°C. Enzyme activity in the assay medium contained 10mM Tris/HCl, pH 8.0, 10mM 5,5′dithiobis- (2 nitrobenzoic acid) (DTNB), 30mM acetyl Coenzyme A (Acetyl CoA) and 10mM oxaloacetic Acid (OAA; omitted for the control) in a final volume of 0.2 ml. Citrate synthase catalyzes the reaction between acetyl CoA and OAA to form citric acid. The increase in O.D. at 412nm was measured in a thermo Scientific Multiskan at 25°C. Enzyme activity was calculated using an extinction coefficient of 13.6 mM⁻¹cm⁻¹at 412nm. We calculated mean mass-specific activities as µmol min⁻¹ g fresh mass¹, mean total activity (mass-specific activity per wet muscle mass) as µmol min⁻¹ and specific activity per gram of protein. Because mass-specific and specific activity per gram of protein were all highly correlated (all r² > 0.7 and P < 0.05) and the results of statistical analysis were similar, we only report total and mass-specific activities for each tissue.

1.4 Statistical analyses

To check if our data were normally distributed, we performed a Kolmogorov-Smirnov test followed by Lilliefors and Levene's test to assess the homogeneity of variances. For variables which were not normally distributed (e.g., BMR and thermal conductance), we used logarithmic transformation for statistical analysis. We evaluated statistically enzyme activities for each tissue in cold and warm acclimated birds by ANOVA using acclimation condition as single factor. A similar analysis was performed for the seasonal study. Except for C values in the seasonal study, BMR and C were positively and significantly correlated with body mass. We tested the homogeneity of slopes to determine whether a traditional ANCOVA or a separate slope design should be used. The homogeneity of slopes model analyses revealed that all treatment by covariate interactions were no significant (all P > 0.2, see below). Then we performed an ANCOVA to compare BMR and C between treatments using Log BMR and log C as dependent variables and log initial body mass as a covariate. To compare differences in C between seasons, we performed an ANOVA test. Since birds were exposed for the same time and Tₐ's inside the metabolic chambers, we assumed that mass loss was similar for all birds in the study. For each treatment we evaluated the potential associations between physiological, morphological and biochemical variables by means of Pearson correlations. In cases when morphological and physiological variables were correlated with body mass, we also used the residuals of those variables against body mass to perform the correlation analysis. In addition, lineal regression analyses between the proportion of CS activity in the pectoral muscle and in legs (CSₚ / CSₗ) and between physiological variables were performed. We also evaluated the association between the CS and COX activities in the same tissue. Statistical analyses were per-
formed using the STATISTICA® (1997) statistical package for Windows. Data are reported as mean ± SE. The numbers of cases for each variable may differ because some tissues were lost and we were unable to obtain measurements of thermal conductance in all birds.

2 Results

2.1 Seasonal acclimatization in the field

2.1.1 Basal metabolic rate and thermal conductance

Basal metabolic rate in summer birds (57.15 ± 4.8 mL O₂/h) was 28% higher than that of winter birds (44.7 ± 4.9 mL O₂/h), although this difference was only close to significance (ANCOVA \( F_{1,17} = 3.82, \ P = 0.067 \); Parallelism: \( F_{1,15} = 1.05, \ P = 0.32 \)). A similar result was found for \( C \) (0.520 ± 0.146 and 0.428 ± 0.166 mL O₂ g⁻¹ h⁻¹ °C⁻¹ for summer and winter birds, respectively; ANCOVA \( F_{1,16} = 1.218, \ P = 0.28 \); Parallelism: \( F_{1,15} = 1.27, \ P = 0.28 \)). No differences were found between body masses of winter and summer birds (Table 1). We observed that small intestine and gizzard masses increased in winter by about 28% (\( F_{1,18} = 7.75, \ P = 0.012 \) and \( F_{1,18} = 8.84, \ P = 0.008 \), respectively). Also, the flight muscle was 30% heavier in summer than in winter (\( F_{1,17} = 4.99, \ P = 0.040 \)). The masses of liver (\( F_{1,18} = 0.66, \ P = 0.536 \)) and heart (\( F_{1,18} = 2.98, \ P = 0.592 \)) remained statistically stable between summer and winter (Table 1). When all field data were pooled, we found a significant correlation between the mass of flight muscles and BMR (\( r = 0.534, \ P = 0.024 \)). A similar result was observed when the residuals of flight muscles with the residuals of BMR were correlated (\( r = 0.47, \ P = 0.040 \)). No significant effect of liver mass on BMR was observed (\( r = 0.16, \ P = 0.526 \) and \( r = 0.05, \ P = 0.850 \) for standard and residual analysis respectively).

2.1.2 Metabolic enzymes

No significant differences in the activity of metabolic enzymes between seasons were observed for any tissue, both for total and per gram of wet tissue (Table 2).

Table 1  Body mass and wet mass (mean ± SE) of organs of Zonotrichia capensis for two seasons

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>Summer</th>
<th>Winter</th>
<th>( F  )</th>
<th>df</th>
<th>( P  )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>10</td>
<td>18.614 ± 0.626</td>
<td>19.374 ± 0.576</td>
<td>0.001</td>
<td>1,16</td>
<td>0.973</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>10</td>
<td>0.539 ± 0.036</td>
<td>0.635 ± 0.064</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine (g)</td>
<td>10</td>
<td>0.498 ± 0.146</td>
<td>0.428 ± 0.166</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>8</td>
<td>0.251 ± 0.005</td>
<td>0.257 ± 0.009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gizzard (g)</td>
<td>10</td>
<td>0.737 ± 0.030</td>
<td>0.948 ± 0.064</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Heart (g)</td>
<td>10</td>
<td>0.23 ± 0.011</td>
<td>0.22 ± 0.015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flight muscles (g)</td>
<td>9</td>
<td>1.664 ± 0.119</td>
<td>1.271 ± 0.127*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Asterisks denote significant differences (\( P < 0.05 \)) after ANOVA.

Table 2  Enzyme activities (mean ± SE) of citrate synthase (CS) and cytochrome oxidase (COX) of flight muscles and liver of Z. capensis in two seasons from a Mediterranean zone of Central Chile

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Summer (mean ± SE)</th>
<th>Winter (mean ± SE)</th>
<th>( F  )</th>
<th>df</th>
<th>( P  )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver CS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/min</td>
<td>9</td>
<td>3.602 ± 0.999</td>
<td>3.646 ± 0.825</td>
<td>0.001</td>
<td>1,16</td>
<td>0.973</td>
</tr>
<tr>
<td>µmol/min mg protein</td>
<td>9</td>
<td>0.768 ± 0.177</td>
<td>1.106 ± 0.389</td>
<td>0.022</td>
<td>1,16</td>
<td>0.883</td>
</tr>
<tr>
<td>µmol/min g</td>
<td>9</td>
<td>6.457 ± 1.688</td>
<td>6.833 ± 1.868</td>
<td>0.623</td>
<td>1,16</td>
<td>0.441</td>
</tr>
<tr>
<td>Liver COX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/min</td>
<td>9</td>
<td>0.759 ± 0.139</td>
<td>0.615 ± 0.194</td>
<td>0.364</td>
<td>1,16</td>
<td>0.555</td>
</tr>
<tr>
<td>µmol/min mg protein</td>
<td>9</td>
<td>0.164 ± 0.040</td>
<td>0.171 ± 0.073</td>
<td>0.008</td>
<td>1,16</td>
<td>0.928</td>
</tr>
<tr>
<td>µmol/min g</td>
<td>9</td>
<td>1.409 ± 0.283</td>
<td>0.978 ± 0.279</td>
<td>1.178</td>
<td>1,16</td>
<td>0.293</td>
</tr>
<tr>
<td>Flight muscles CS</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>µmol/min</td>
<td>9</td>
<td>13.061 ± 2.249</td>
<td>13.360 ± 3.036</td>
<td>0.006</td>
<td>1,17</td>
<td>0.939</td>
</tr>
<tr>
<td>µmol/min mg protein</td>
<td>10</td>
<td>0.878 ± 0.111</td>
<td>1.033 ± 0.190</td>
<td>0.470</td>
<td>1,17</td>
<td>0.502</td>
</tr>
<tr>
<td>µmol/min g</td>
<td>9</td>
<td>8.243 ± 1.753</td>
<td>10.020 ± 1.889</td>
<td>0.469</td>
<td>1,17</td>
<td>0.503</td>
</tr>
<tr>
<td>Flight muscles COX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/min</td>
<td>9</td>
<td>2.161 ± 0.332</td>
<td>2.506 ± 0.627</td>
<td>0.237</td>
<td>1,16</td>
<td>0.633</td>
</tr>
<tr>
<td>µmol/min mg protein</td>
<td>8</td>
<td>0.284 ± 0.035</td>
<td>0.315 ± 0.081</td>
<td>0.110</td>
<td>1,15</td>
<td>0.745</td>
</tr>
<tr>
<td>µmol/min g</td>
<td>9</td>
<td>1.402 ± 0.258</td>
<td>1.984 ± 0.525</td>
<td>0.989</td>
<td>1,16</td>
<td>0.335</td>
</tr>
</tbody>
</table>
2.2 Laboratory experiments

2.2.1 Basal metabolic rate

Basal metabolic rate was ca 20 % higher in cold-acclimated birds than in warm acclimated birds (ANCOVA $F_{1,16} = 7.374, P=0.01$; Parallelism: $F_{1,15} = 1.24, P = 0.28$, Fig. 1A). We also found a significant increase in thermal conductance in animals acclimated to the warm compared to those acclimated to the cold treatment (ANOVA $F_{1,17} = 4.86; P = 0.041$, Fig. 1B). After six weeks of thermal acclimation, we observed no significant effect of the treatment on body mass or on the wet mass of internal organs, flight muscles or mixed leg muscles (see Table 3 for mean values and statistics). We observed a significant and positive effect of the mass of flight muscles ($r = 0.48, P = 0.035$) on BMR. Besides, we found a trend toward an increase in BMR with an increase in liver mass ($r = 0.41, P = 0.07$). However, these associations weakened when mass-independent residuals were used ($r = 0.41, P = 0.07$ and $r = 0.35, P = 0.14$, for flight muscles and liver respectively).

2.2.2 Metabolic enzymes

After six weeks of treatment, a significant treatment effect was detected on total CS activity of flight muscles ($F_{1,17} = 5.40, P = 0.032$). The analysis revealed that this activity was higher in the flight muscles of cold-acclimated than in warm-acclimated birds (Fig. 2). The analysis also revealed a similar effect of treatment on CS activity per gram of wet tissue ($F_{1,17} = 4.79, P = 0.042$). Total and CS activity per gram of wet tissue of liver were similar between acclimation treatments ($F_{1,17} = 0.488, P = 0.494$ and $F_{1,17} = 0.858, P = 0.367$ respectively). Total leg muscle CS activity was higher in the warm than in the cold group, although the difference was not significant ($F_{1,17} = 3.118, P = 0.093$). No significant differences in CS activity per gram of wet leg muscle were found ($F_{1,17} = 2.449, P = 0.135$).

COX activity in the liver was unaffected by thermal treatment ($F_{1,17} = 0.010, P = 0.924$ and $F_{1,17} = 0.022, P = 0.884$, for total activity and activity standardized per gram of wet tissue, respectively). A similar result was found for total activity in the flight muscles ($F_{1,17} = 0.649, P = 0.631$), as well as per gram of wet tissue ($F_{1,17} = 0.658, P = 0.428$). Leg muscle COX activity was also similar between thermal treatments ($F_{1,17} = 2.051, P =$

![Fig. 1 Basal metabolic rate (A) and thermal conductance (B) in cold- and warm-acclimated individuals of Zonotrichia capensis](image)
P-values indicate significant effects of the treatment after ANCOVA.

| Table 3 Body mass and mass of internal organs, flight muscles and mixed leg muscles (means ± SE) of Zonotrichia capensis acclimated to 15 °C and 30 °C for six weeks |
|---------------------------------------------|----------------|----------------|----------------|----------------|----------------|
|                | n  | 30°C         |             | 15°C          | F_{1,17}     | P   |
| Body mass (g)  | 10 | 18.16 ± 0.501 | 9            | 17.822 ± 0.418 | 0.261        | 0.616 |
| Liver (g)      | 10 | 0.61 ± 0.029  | 9            | 0.59 ± 0.035  | 0.186        | 0.672 |
| Intestine (g)  | 10 | 0.615 ± 0.026 | 9            | 0.57 ± 0.045  | 0.877        | 0.362 |
| Cloaca (g)     | 10 | 0.081 ± 0.006 | 9            | 0.074 ± 0.007 | 0.627        | 0.439 |
| Kidney (g)     | 9  | 0.197 ± 0.008 | 8            | 0.214 ± 0.007 | 2.541*       | 0.132 |
| Gizzard (g)    | 10 | 0.714 ± 0.023 | 9            | 0.792 ± 0.048 | 2.248        | 0.152 |
| Heart (g)      | 10 | 0.233 ± 0.009 | 9            | 0.262 ± 0.030 | 0.924        | 0.350 |
| Flight muscles (g) | 10 | 1.755 ± 0.150 | 9            | 1.753 ± 0.085 | <0.001       | 0.993 |
| Leg muscles (g) | 10 | 1.343 ± 0.061 | 9            | 1.231 ± 0.080 | 1.273        | 0.275 |

ANOVA F and P are also shown. *Degrees of freedom for kidney analysis were (1,15).
0.170 and $F_{1,17} = 0.189, P = 0.669$, for total activity and activity standardized per gram of wet tissue respectively, Fig. 2). Finally, we found a significant variation in the $\text{CSp/CS}_1$ ratio between the two experimental groups, both for total and mass-specific bases ($F_{1,17} = 13.093, P = 0.002$ and $F_{1,17} = 6.072, P = 0.025$, respectively). In both cases, this ratio was higher in cold-acclimated compared to warm-acclimated birds. No difference in this ratio was found for COX activity ($F_{1,17} = 0.236, P = 0.634$, $F_{1,17} = 0.037, P = 0.851$ in total and mass-specific activities, respectively). Finally we found a weak but significant and positive association between basal metabolic rate and total CS activity in pectoral muscle ($r^2 = 0.217, P = 0.044$) and with the $\text{CSp/CS}_1$ ratio ($r^2 = 0.290, P = 0.017$; Fig. 3). Also we observed that thermal conductance correlated positively with the CS activity of lower limbs ($r^2 = 0.40, P = 0.003$) and negatively with the $\text{CSp/CS}_1$ ratio. In this last case an exponential model was the best fit to our data set ($r^2 = 0.48, P = 0.005$, Fig. 3C).

3 Discussion

3.1 Seasonal acclimatization

The results revealed a lack of seasonal variation in BMR and thermal conductance, at least during the months we sampled, although BMR in summer birds exceeded that in winter birds by 28%, a difference that approached significance. This trend is also consistent with summer birds having 30% larger pectoralis muscles than winter birds. In addition, no changes were reported between February (summer) and July (winter) in the activity of enzymes associated with aerobic metabolism. Our results are comparable with at least two previous studies in which seasonal energetics of $Z. \text{capensis}$ have been evaluated in similar Mediterranean environments. First, Novoa (1993) documented monthly variation of energy expenditure in this species, detecting only a significant increase of BMR during summer, and Maldonado et al. (2009), reported no changes in BMR between seasons. Differences in the BMR response to seasonality among years in $Z. \text{capensis}$ agrees with previous results in three bird species in which metabolic rates (including BMR) varied within and among winters (Swanson and Olmstead, 1999). Furthermore, several studies have suggested that an increase in the rates of energy intake and expenditure during cold exposure would not be universal, and a winter decrease in these rates may also occur (Gelineo, 1969; Lustick et al., 1982; Dawson et al., 1983; McKechnie, 2008, Dawson and O’Connor, 1996). Thus the increase of BMR in winter would not be a requirement for survival in winter, and the acclimatization of BMR would be a species-specific response (Swanson, 2010; van de Ven et al., 2013). In this vein, although it has been suggested that winter increases in BMR are correlated with cold resis-
tance in birds, the functional significance of this seasonal variation in meeting the energetic demands of thermogenesis is still controversial (see Swanson, 2010; Petit et al., 2013).

Recently, Smit and McKechnie (2010) reported that mass-specific BMR in Kalahari Desert bird species were significantly lower in winter than in summer. This contrasts with the typical acclimatization response of birds from higher latitudes. Also, at an interspecific level and global scale, Smit and McKechnie (2010) analyzed the pattern of variation of winter and summer BMR acclimatization, finding that both the direction and magnitude of seasonal BMR changes varied geographically. In fact winter/summer mass-specific BMR ratios decreased as the mean winter temperatures of acclimatization sites increased. Results in *Z. capensis* agree with the pattern identified by Smit and McKechnie (2010), in which birds inhabiting sites with a mean temperature of the coldest month near 15°C should exhibit no change in BMR between summer and winter (see van de Ven, 2013, for an example in an African subtropical passerine). Thus, the moderate difference in minimum temperatures between seasons in Mediterranean environments would be not enough to trigger seasonal adjustments in BMR of bird species.

This study revealed constancy between summer and winter in both tissues for enzyme activity in *Z. capensis* inhabiting a Mediterranean environment, which is consistent with the absence of changes in BMR. Evidence about seasonal adjustments of citrate synthase activity in Passeriformes is not conclusive. In fact some studies have reported a lack of seasonal variation in mass-specific activity of CS in pectoral muscle and heart (Marsh and Dawson, 1982; Yacoe and Dawson, 1983; Carey et al., 1989; O’Connor, 1995; Vézina and Williams, 2005; Liknes and Swanson, 2011; this study), and there are cases of variation between seasons (Liu et al., 2008; Zheng et al., 2008, 2010; Liknes and Swanson, 2011). In addition, we did not find seasonal differences in the enzymatic activities of CS and COX from the liver, a result that contrasts with those reported by Zheng et al. (2008), who found a winter increase in liver COX of *Passer montanus*. These documented differences in the response of metabolic enzymes depend on the species, tissue, and enzyme being studied. Contrary to expectations, those species and populations exhibiting higher activities in metabolic enzymes in winter than in summer (e.g., Liu et al., 2008; Liknes and Swanson, 2011) do not dwell in habitats whose seasonal thermal variation is higher compared with those inhabiting less thermally variable environments (Yacoe and Dawson, 1983; Vézina and Williams, 2005). This fact suggests that seasonal variation in metabolic enzymes may also be related to other factors not related with temperature, such as contractile activity in skeletal muscle, which is intensified during exercise or migration (Marsh, 1981;
Lundgren and Kiessling, 1985; Lundgren and Kiessling, 1986; Lundgren and Kiessling, 1988; Butler and Turner, 1988; Hammond et al., 2000). Another potential factor affecting metabolic enzymes could be photoperiod, which has been demonstrated to induce changes in fatty acid transporters in skeletal muscle in Zonotrichia albicollis (Zajac et al., 2011). This potential source of variation in enzyme activities deserves further attention in species inhabiting seasonal environments; especially those facing moderate fluctuations in abiotic conditions between different seasons.

3.2 Thermal acclimation

Patterns of metabolic variation differed considerably between seasonal and acclimation experiments. As mentioned above, the magnitude of a flexible response of BMR seems to be dependent on the intensity of the environmental cues. It is generally considered that BMR is primarily driven by temperature, although it is possible that other factors such as photoperiod, reproduction, and body condition may be influencing BMR in the field (Daan et al., 1990; Vézina and Williams, 2005; Chastel et al., 2003; Zheng et al., 2008; McNab, 2009; Vézina and Salvante, 2010). Several studies have shown that thermal acclimation can affect BMR in birds, increasing after cold exposure and decreasing after acclimatization to high temperatures (Gelineo, 1964; McKechnie, 2008). Our laboratory results support this idea. Coupled with changes in BMR, cold-acclimated Z. capensis exhibited an increase in the activity of CS in the pectoral muscle (Fig. 2). Moreover, we found a significant and positive relationship between BMR and CS activity in the flight muscles (Fig. 3A). Thus our results support the idea that changes in oxidative activity in tissues with thermogenic activity may lead to changes in the rate of energy consumption of the whole organism. Such trends are consistent with the results reported by Liknes and Swanson (2011) who found significant increases in oxidative activity of CS in the pectoral muscle in birds acclimatized to winter climate. Recently, Zheng et al. (2013) reported that Chinese bulbuls Pycnonotus sinensis exhibited higher COX activities both in liver and muscle after being acclimated for 4 weeks to 10 °C compared to those acclimated to 30 °C. They also found that BMR was positively correlated with COX activity in the liver and muscle. Although we found a significant and positive correlation between COX and CS activities in the flight muscles of acclimated birds, the groups had similar COX activities among treatments. Unfortunately, the study of Zheng et al. (2013) did not report CS activity, which does not allow establishing whether both enzymatic changes are involved in the variation in BMR and prevents a proper comparison with this study. However, the results of our study suggest that even though the response of BMR to thermal acclimation may be common in birds, the biochemical mechanisms involved in power generation may differ between species. Some authors have suggested that BMR mainly depends on the metabolic intensity and the mass of internal organs such as liver and kidneys, whereas skeletal muscles are mainly related to maximum metabolism ($M_{\text{max}}$) (Hoppeler and Weibel, 1998; Swanson, 2010; Zheng et al., 2013). The positive association observed between mass of the pectoral muscle, enzyme activity and BMR, suggested that skeletal muscle may also affect the level of BMR, a result consistent with the data of Chappell et al. (1999).

Studies assessing the activity of metabolic enzymes in Passeriformes have generally focused on the activities of pectoral muscle and liver; however, the oxidative capacity of leg muscles has received less attention. Studies oriented to measure the activity of these two muscle groups have reported that the proportion CS<sub>p</sub>/CS<sub>i</sub> is close to 3 (Marsh and Wickler, 1982; Marsh and Dawson, 1982; Carey et al., 1989; Choi et al., 1993; Olson, 2001; Dawson and Olson, 2003). In this study the average value for CS<sub>p</sub>/CS<sub>i</sub> in cold-acclimated birds was 3.3, and 1.5 for warm-acclimated birds. Thus birds having higher rates of energy expenditure due to higher thermoregulatory requirements increased oxidative activity in the pectoral muscles relative to leg muscles. In addition, when all experimental birds were pooled, BMR was positively correlated with activity of citrate synthase in the flight muscles and with the CS<sub>p</sub>/CS<sub>i</sub> ratio. This result reveals modulation of catabolic pathways within individual birds, depending on the thermoregulatory demands. Thus, in Zonotrichia capensis the main role of thermogenesis can be attributed to the pectoral muscles, while muscles located distally (e.g., gastrocnemius complex) do not seem to play an important role.

Furthermore, our results revealed a positive relationship between C and CS enzymatic activity of muscles of the lower limbs and a negative and non-linear correlation between C and the CS<sub>p</sub>/CS<sub>i</sub> ratio (Fig. 3). In this last case C seems to decrease with CS<sub>p</sub>/CS<sub>i</sub> ratio until a steady stable ratio is reached, suggesting the existence of a limit for a decrease in thermal conductance. Some authors have suggested that lower limb muscles could be recruited only secondarily for thermogenesis in birds (e.g. Carey et al., 1989; Olson, 1994; Dawson and Olson, 2003), but others suggested that muscles associated
with flight (pectoralis major and supracoracoideous) and lower appendicular musculature could be decisive for shivering thermogenesis (Vittoria and Marsh, 1996; Duchamp and Barré, 1993; Marsh and Dawson, 1989; Carey et al., 1989; Aulie and Toien, 1988). We propose that the oxidative activity of the lower extremities in the sedentary condition (i.e., birds restricted from flying), actively participates in the thermoregulatory process. Thus, if birds are acclimated below their TNZ, flight muscles increases their catabolic capacity, allowing heat generation in a wider area of the body, properly isolated and close to the internal organs. In addition, the constancy in oxidative activity in legs could produce a temperature gradient between the body core and the appendages lacking adequate thermal insulation, which is consistent with the reported presence of regional heterothermy generated by vascular countercurrent mechanisms in birds exposed to cold conditions (Chattfield et al., 1953). Therefore the reported regional heterothermy in lower limbs could be due to the reduced body heat gradient generated by the countercurrent system, but also may be due to differences in oxidative metabolic activity in situ among tissues.

But, why do physiological and underlying biochemical traits vary in the laboratory but not in the wild? As pointed out by McKechnie and Swanson (2010), the links between metabolic responses to thermal acclimation and seasonal acclimatization remain unclear because of observed divergent BMR responses of birds in the field and due to thermal acclimation. In this vein, the absence of seasonal acclimatization in BMR does not necessarily reflect environmental stability or an absence of physiological flexibility. Indeed, this study reveals the absence of metabolic and biochemical adjustments between winter and summer in the passerine Z. capensis, suggesting that behavioral but not physiological adjustments are used by some bird species to face moderate changes in environmental conditions in Mediterranean zones. Another example of differences between BMR acclimatization and acclimation was recently reported for two populations of southern red bishops Euplectes orix (van de Ven et al., 2013a,b). These authors found that inland and coastal populations of this species had a similar responses in BMR to thermal acclimation (van de Ven et al., 2013a), but showed divergent patterns of seasonal acclimatization. In fact, total BMR of wild bishops from a coastal site remains similar between seasons but red bishops from an inland site showed a 58% increases in total BMR in Winter (van de Ven et al., 2013b). These authors suggested that seasonal changes that occur during acclimatization are driven by factors other than ambient temperature alone. We agree with that and hypothesized that most species have the potential for physiological and biochemical flexibility, but this is not expressed in the field when animals can buffer environmental variability through regulatory behavior. In the laboratory, animals cannot avoid thermal conditions, and hence the only adaptive response available is to modify the energy expenditure. Thus, it is probable that the use of micro-site selection and behavioral traits, such as food caching, cavity roosting, and changes in activity patterns by Z. capensis, could be a successful strategy to reduce the energetic demands associated with winter temperatures. For example, it has been reported that birds can respond to variation in heat exchange by changing foraging behavior (Morton 1967; DeWoskin 1980). Furthermore, there is another potential driver of seasonal adjustments in BMR. As we already discussed, it is commonly believed that BMR is primarily driven by temperature. Nevertheless, in winter months the increase of energy expenditure due the low environmental temperature requires a reliable supply of food. In this sense, increases in metabolic rates in coldest months necessarily rely upon the capacity to obtain food to supply the higher energy demands imposed by the environment for thermoregulation (e.g., Goymann et al. 2006). In this sense, individuals which have been found to decrease their BMR in winter, inhabits regions characterized by the presence of dry and unproductive winters (see Smit and McKeenie, 2010) raising the possibility that the temporal dynamic of BMR changes could be affected by the interplay between temperature and resource availability at the intraspecific (e.g., seasonal) or even at interspecific level. Considering the Mediterranean habitats of central Chile, these exhibit seasonal variations in the availability of food, especially insects (Arroyo et al. 1985, Simonetti 1989). In fact, López-Calleja (1995) reported significantly decrease in insect availability during winter in the study area. In this regard, the apparent trend towards a decrease in BMR in winter in Z. capensis seems to support such hypothesis.

In conclusion, it appears that for birds inhabiting environments with Mediterranean climates a regulatory behavioral response may be sufficient for coping with the relatively minor seasonal variation in climatic conditions. Indeed, as suggested by Brandon (1988), organisms can damp out abiotic environmental variability through regulatory behavior. In contrast, when winter conditions are more severe, additional physiological and
biochemical adaptive response are required. In fact, physical environments are not constant and consequently organisms can undergo fluctuations that may differ in time scales and severity (Petit el al., 2013). In this case, large-scale climate oscillations might maintain the flexibility in thermogenic traits through generations.

Acknowledgements This work was financed by Fondo Nacional de Desarrollo Científico y Tecnológico (Chile Grant no. 1120276) to P Sabat. Birds were captured with permits from SAG, Chile (No. 840/2012). All protocols were approved by the Institutional Animal Care Committee of the Universidad de Chile, where the experiments were performed. We thank the Associate Editor, David Swanson and two anonymous referees for their useful comments on an earlier version of the manuscript. We also thank Andres Sazo and Natalia Ramirez for their invaluable help in the field and laboratory.

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