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Analysis of subcellular energy metabolism in five Lacertidae lizards across varied environmental conditions

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ABSTRACT

Aerobic respiration is the main energy source for most eukaryotes, and efficient mitochondrial energy transfer greatly influences organismal fitness. To survive environmental changes, cells have evolved to adjust their biochemistry. Thus, measuring energy metabolism at the subcellular level can enhance our understanding of individual performance, population dynamics, and species distribution ranges. We investigated three important metabolic traits at the subcellular level in five lacertid lizard species sampled from different elevations, from sea level up to 2000 m. We examined hemoglobin concentration, two markers of oxidative stress (catalase activity and carbonyl concentration) and maximum rate of metabolic respiration at the subcellular level (potential metabolic activity at the electron transport system). The traits were analysed in laboratory acclimated adult male lizards to investigate the adaptive metabolic responses to the variable environmental conditions at the local sampling sites. Potential metabolic activity at the cellular level was measured at four temperatures – 28 °C, 30 °C, 32 °C and 34 °C – covering the range of preferred body temperatures of the species studied. Hemoglobin content, carbonyl concentration and potential metabolic activity did not differ significantly among species. Interspecific differences were found in the catalase activity, Potential metabolic activity increased with temperature in parallel in all five species. The highest response of the metabolic rate with temperature (Q_{10}) and Arrhenius activation energy (E_a) was recorded in the high-mountain species *Iberolacerta monticola*.

1. Introduction

Aerobic respiration is the primary source of energy for most eukaryotes (Berg et al., 2019). Traditional ecological methods for quantifying energy metabolism have estimated metabolic rates by measuring gas exchange in whole organisms (Glazier, 2015) and this approach has greatly improved our understanding of variation in individual performance, population growth rates and community structures (Brown et al., 2004). However, respiration at the whole organism level ultimately derives from energy flow at the subcellular level (e.g. Delhaye et al., 2016), with aerobic respiration in the mitochondria being a central process by which organisms transfer energy from substrates into the chemical bonds of adenosine triphosphate (ATP) (Berg et al., 2019).

The efficiency of mitochondrial energy transfer can be a major determinant of organismal fitness (Hill et al., 2019; Heine and Hood, 2020). Moreover, to survive in a changing world, cells evolved mechanisms for adjusting their biochemistry in response to signals indicating environmental change (Somero et al., 2017). The adjustments can take many forms, including changes in the activities of preexisting enzyme molecules, changes in the rates of synthesis of new enzyme molecules, and changes in membrane-transport processes (Somero et al., 2017). Looking at subcellular-level processes of aerobic respiration can thus serve as indicator of adaptive or plastic changes needed for organisms to respond to a changing environment.

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Maintaining energy demands and preventing harmful byproducts of aerobic respiration requires precise mitochondrial coordination (Berg et al., 2019). Electrons flow through the electron transport chain (ETC), pumping protons into the intermembrane space, creating a charge imbalance. When electrons escape the ETC and react directly with oxygen there is production of reactive oxygen species (ROS) that have essential signalling functions (Berg et al., 2019). However, if the rate of ROS production is higher than the capacity of antioxidant systems, this can cause oxidative damage and cellular dysfunction (Hermes-Lima, 2004). The cellular defence strategies against oxidative damage by ROS are through enzyme-mediated reactions that catalyse the conversion of superoxide radicals into hydrogen peroxide and molecular oxygen (Berg et al., 2019). Catalase is responsible for dismutation of the hydrogen peroxide into water and molecular oxygen (Berg et al., 2019). These processes are also termed antioxidative processes or defence mechanisms. Since production of ROS is typically increasing with metabolic rate and is constrained by antioxidant defences, only investigating both metabolic rate and antioxidant defence mechanisms at the same time will provide us with information about potential harmful oxidative stress (Somero et al., 2017; Koch et al., 2021).

Hemoglobin is a vital protein in red blood cells that is responsible for transporting oxygen from the lungs to the tissues and returning carbon dioxide from the tissues to the lungs for exhalation (Alberts et al., 2015). Its main role in aerobic respiration is to provide the oxygen needed for the cellular processes that produce ATP (Perutz, 1970). The efficiency of oxygen delivery by hemoglobin is influenced by its structure, oxygen affinity and concentration (Natarajan et al., 2016) as well as environmental factors such as altitude, temperature, and oxygen partial pressure (e.g. Lutz and Bentley, 1985; Gangloff et al., 2019; Megía-Palma et al., 2020a; Pu et al., 2024). Variations in hemoglobin between species illustrate the diverse evolutionary strategies employed to optimize oxygen transport and utilization in response to specific environmental and physiological demands.

Energy from aerobic metabolism is derived from oxidation reactions and these biochemical processes are strongly temperature dependent (Ritchie, 2018). The rate at which oxygen consumption increases with a rise in body temperature is similar in endotherms (Schmidt-Nielsen, 1997; Clarke, 2017) and ectotherms, particularly lizards (Angilletta Jr., 2009). The difference to endotherms, however, is that the body temperatures of ectotherms, such as lizards, depend on external heat sources (Huey and Kingsolver, 1989). Thus, of the factors that can influence metabolic performance in ectothermic animals (i.e. body size, sex, reproductive and nutritional status, seasonal activity), body temperature is the most influential (Bennett and Dawson, 1976). It is beneficial for ectotherms to maintain body temperature within the narrow temperature range in which metabolic performance is optimised (Angilletta Jr., 2009). Moreover, an increase in metabolic rate may be associated with energetic costs, so that under certain environmental conditions (e.g. lack of trophic resources, extreme temperatures) it may be equally optimal to decrease metabolic rate (Huey and Kingsolver, 1989). Lizards' thermal preferences are to some extent conserved in the phylogeny of some groups (Garcia-Porta et al., 2019), but may also display substantial plasticity across species inhabiting heterogeneous environments (e.g. Bauwens et al., 1995; Angilletta Jr. et al., 2010; Grigg and Buckley, 2013; Hertz et al., 2013; Megía-Palma et al., 2020a, 2020b; Serén et al., 2023). In principle, we would expect lizards with similar preferred body temperatures to have similar thermal optima of metabolic performance.

To improve our understanding of how mitochondrial phenotypes are expressed under current environmental conditions, our study focused on the comparison of different subcellular aspects of metabolism in five selected lizard species of the family Lacertidae with variable ecology and distribution range, but relatively similar thermal preferences. We sampled four species of *Podarcis* and one species of *Iberolacerta* at four different sites in Portugal. The sites differed in elevation (from 2 to 1990 m above sea level) and climate. Higher altitudes are generally colder and represent more thermally restrictive environments for lizards (e.g. Aguado and Braña, 2014; Ortega et al., 2017). Phylogenetically, four species of the same genus (*Podarcis*) are closely related and share a common ancestor from the late Miocene (Yang et al., 2021), while *Iberolacerta* belongs to a more distant genus but within the tribe Lacertinii and separated from the genus *Podarcis* about 35 M years ago (Garcia-Porta et al., 2019). We examined hemoglobin concentration as the oxygen-limiting delivery system for aerobic respiration. Next, we determined two markers of oxidative stress, namely catalase activity and carbonyl concentration and we estimated the potential metabolic activity (PMA; an estimate of the maximum enzymatic potential of the mitochondrial respiratory chain). Our prediction was that traits at the subcellular level of metabolism will vary across species, but we might find similarities in metabolic traits among those species originating from similar altitudes and environments since those species also exhibit closest thermal preferences.

2. Materials and methods

2.1. Study design and lizard species

We investigated physiological parameters related to metabolism in five lizard species of the family Lacertidae sampled in the Iberian Peninsula: *Iberolacerta monticola*, *Podarcis carbonelli*, *Podarcis virescens*, *Podarcis siculus* and *Podarcis lusitanicus*. *Iberolacerta monticola* is endemic to two separate sub-areas in Iberia: the Cantabric range and Serra da Estrela (Martín, 2015); *P. carbonelli* has a fragmented range that includes coastal sites in the centre and southwest, as well as some mountain ranges in central Iberia; *P. virescens* occurs in the central and southern parts of the Iberian Peninsula; *P. lusitanicus* is restricted to NE Iberian Peninsula (Sillero et al., 2014; Caeiro-Dias et al., 2018); *P. siculus* is an introduced species in the Iberian Peninsula (Oskyrko et al., 2022), while it naturally occurs in the Italian Peninsula, large islands of the central Mediterranean and the adjacent Adriatic coast, and the Balkans (Sillero et al., 2014). Adults of all species are relatively small, with an average length between snout and vent (SVL) of 5.0 to 7.3 cm and mass of 3 to 10 g (Speybroeck et al., 2016; Table 1).

Only male lizards were used in this study because both sexes can exhibit different eco-physiologies (e.g. Carretero et al., 2005; Liwanag et al., 2008; Naya et al., 2008). Lizards were collected at four different localities and at different elevations ranging from 2 to 1990 m a.s.l (Fig. 1, Table 1). Lizards were collected in autumn 2020; that is after the breeding season, when they are expected to have recovered fat reserves and to attain optimal body condition (Carretero, 2006). They were kept in the laboratory under the same standard conditions (individual terraria with ad libitum water, fed *Tenebrio molitor* dusted with vitamin powder thrice a week, a natural photoperiod provided by a window and infra-red lamp for heating and basking turned on for 10 h each day) for approximately 1 month (Table 1). Keeping the animals in the laboratory for around 1 month is a sufficiently long period to remove the effects of immediate acclimatisation stress and is typical for studying the physiology of reptiles and their responses to environmental changes (e.g. Watson and Burggren, 2016). After lab acclimatisation, they were used in ecophysiological trials and samples for this study were taken after the experiments finished. All lizards were in good body condition at the time of sampling as well as and after samples were collected, which was assessed visually (general physical condition and responsiveness to handling). We measured the snout vent length (SVL) with a ruler to the nearest 1 mm and weighed the lizard with a scale to the nearest 0.1 g. We calculated the body condition index (BCI) by regressing log SVL values against log weight and using the residual values as BCI scores (Table 1).

2.2. Tissue and blood collection

Tail tips were sampled from 9 to 11 males per species (Table 1). A tail tip of maximum 1 cm was collected by gently pulling the tail from its tip,

Table 1
Information on the sample populations including locations (Elev = elevation in m a.s.l., Lat = latitude, Lon = longitude), dates of capture and sample collection, sample size (N), body size (SVL = snout vent length and Weight).

Species	Location of capture	Elev	Lat	Lon	Date of capture	Date of sample collection	N	mean SVL (mm)	SE SVL	mean Weight (g)	SE Weight
Podarcis carbonelli	Santa Maria da Feira	145	40,9206	−85,422	10.10.2020	10.11.2020	10	50,4	0,7	3,1	0,2
Podarcis virescens	Santa Maria da Feira	145	40,9206	−85,422	10.10.2020	10.11.2020	9	57,8	0,6	4,3	0,1
Podarcis siculus	Lisbon	2	38,7179	−91,873	15.09.2020	10.11.2020	9	73,4	0,9	10,1	0,3
Podarcis lusitanicus	Serra de Estrela (Rossim)	1400	40,9206	−85,422	26.09.2020	9.11.2020	11	57,5	1,3	4,8	0,3
Iberolacerta monticola	Serra de Estrela (Torre)	1990	40,3314	−76,169	26.09.2020	9.11.2020	11	70,1	1,1	9,1	0,5

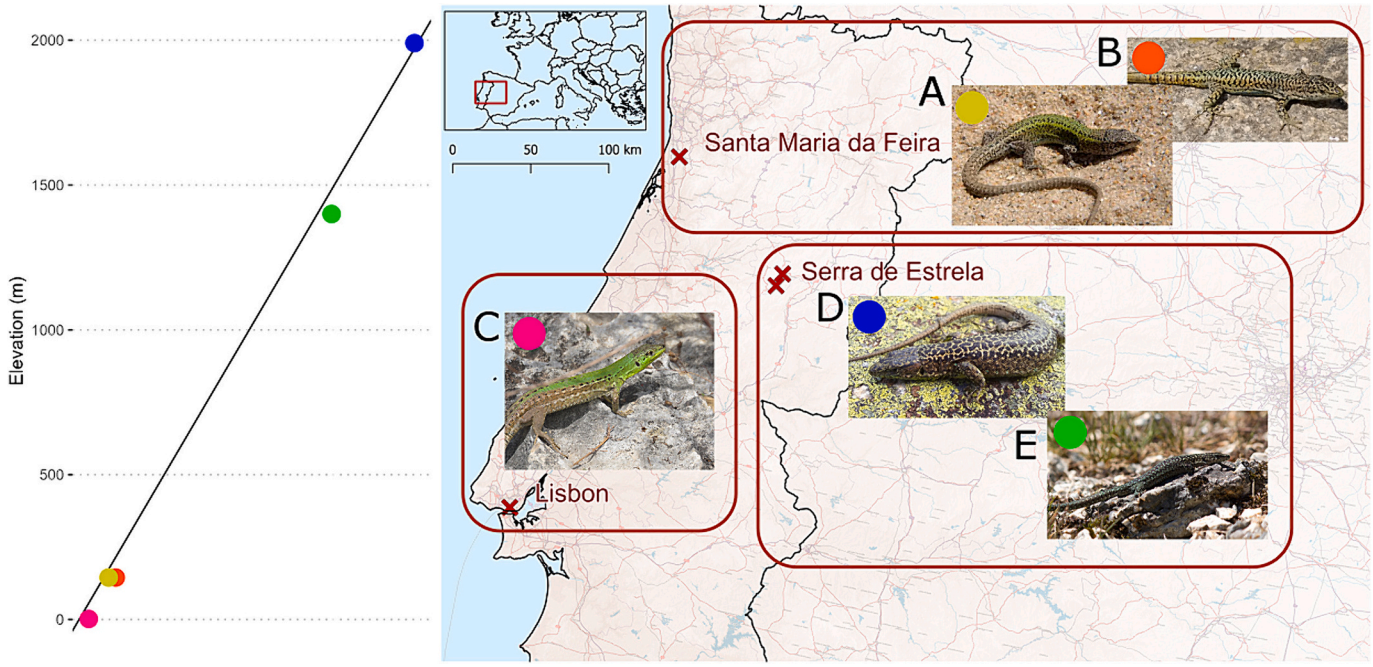

Fig. 1. Sampling locations of five study species with altitude in m above sea level represented on the left side. (A) *Podarcis carbonelli*, (B) *P. virescens*, (C) *P. siculus* (introduced), (D) *I. monticola*, and (E) *P. lusitanicus*.

which released a small tail fragment at a point of natural autotomy. This procedure has already been demonstrated to produce minimal stress and behavioural disturbances (Langkilde and Shine, 2006; García-Muñoz et al., 2011) and carry smaller-scale costs on lizards (Naya et al., 2007). Tail tips were put into empty Eppendorf tubes and immediately stored on dry ice until transferred to the −80 °C freezer, where they were stored until we used them for biochemical analyses. A blood sample (less than 5 μL) was extracted from the tail using sterile needles (BD Microlance 3; 25G; 0.5 × 16 mm). The hemipenes were avoided by sampling at least 2 cm away from the cloaca. Na-heparinized micro capillary tubes were used to collect the blood and quantified total hemoglobin concentration (HB) with an automated device (HemoCue®, Hb 201+, Ängelholm, Sweden) (Megía-Palma et al., 2020a, 2020b). We also measured snout-to-vent length (SVL) and body mass of all lizards, using a ruler (accuracy = 1 mm) and digital scale (accuracy = 0.01 g).

All animal housing and sampling complied with ARRIVE guidelines and was carried out in accordance with EU Directive 2010/63/EU for animal experiments. The work was carried out under permit no. 805-808/2020/CAPT e DET and process reference nr: 41205/2020/DCNB/DAN. AŽ, MAC and RMP are holders of certificate for handling and working with animals under EU Directive 2010/63/EU.

2.3. Biochemical analysis of metabolic markers

The activity at the electron transport system (ETS) level indicates the amount of oxygen consumption that would occur if the multienzyme complexes located in the inner membrane of the mitochondria were to function maximally (Muskó et al., 1995), and we refer to it as the potential metabolic activity (PMA) at the cellular level. In a previous study, it was shown that the ETS activity of a whole animal can be estimated based on the measured ETS activity of a part of an animal body that can be subsequently regenerated (Simčič et al., 2012) and several studies including lizards have shown variation in this metabolic trait in relationship with environmental conditions (e.g. Žagar et al., 2015, 2018a, 2018b, 2022). Antioxidative capacity was estimated using catalase activity (CAT), which serves as a proxy for antioxidant enzyme activity (capacity), and carbonyl protein content (CAR), a widely used marker of oxidative modification of proteins (Suzuki et al., 2010). The tail tips were used for the biochemical assays to determine all three subcellular traits: PMA, CAT and CAR. First, each tail tip was homogenized with liquid nitrogen in a mortar and pestle to obtain finely grinded powder. The ground tissue was weighed to determine the wet mass and used for the homogenate preparation. For PMA and CAT analyses, 3 mL ice-cold homogenization buffer (0.1 M sodium phosphate buffer pH = 8.4; 75 μM

magnesium sulphate; 0.15% (*w/v*) polyvinyl pyrrolidone; 0.2% (*v*/v) Triton-X-100) was added to the grinded tissue. After sonication for 20 s, samples were centrifuged at 0 °C and 8500 ×g for 4 min. First, PMA analysis was performed, and the other half of the homogenate stored at −80 °C until CAT analysis. The remaining part of ground tissue was stored at −80 °C until CAR analysis.

PMA was estimated using the iodonitrotetrazolium chloride (INT) reduction capacity following the protocol from Packard (1971) modified by G.-Tóth (1999), which has already been adapted to lizard tissues (e.g. Žagar et al., 2015, 2018a, 2018b). The INT reduction method is used as a proxy for cellular respiration (Bielen et al., 2016; Žagar et al., 2015), considering that the reduction of INT can also occur extra-mitochondrially and can be associated with cytosolic, lysosomal, and peroxisomal fractions (Berridge et al., 2005; Maldonado et al., 2012). Two replicates of 30 μL samples and one blank, without the homogenate, were all incubated at four different temperatures (28, 30, 32 and 34 °C). These temperatures cover the preferred body temperature range of all the studied species. They were incubated with a 150 μL substrate solution (0.1 M sodium phosphate buffer pH = 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2% (v/v) Triton-X-100) and 50 μL reagent solution (2.5 mM 2-(*p*-iodophenyl)-3-(nitrophenyl)-5-phenyl tetrazolium chloride (INT) solution) for 30 min. After 30 min, the reaction was stopped by adding 50 μL of stopping solution (formalin: concentrated H_3PO_4; 1:1 v/v). Aliquots of 30 μL homogenate were added to the blanks after addition of the stopping solution. Reduction of INT into formazan was determined spectrophotometrically from the absorbance of the sample at 490 nm using a microplate reader (Synergy MX BioTek, USA). The values of PMA were estimated as the INT reduction capacity (μL O_2 mg^{-1} protein h^{-1}) as:

$$INT\ reduction\ capacity = \frac{Abs_{490nm} \times V_r \times V_h \times 60 \times 1.30}{V_a \times S \times t \times 1.42}$$

where $Abs_{490\ nm}$ is the absorbance of the sample (mean value of duplicates minus blank value); V_r is the final volume of the reaction mixture (mL); V_h is the volume of the original homogenate (mL); V_a is the volume of the aliquot of the homogenate; S is the protein mass of sample (mg); t is the incubation time (min); 1.30 is the factor for path length correction (Lampinen et al., 2012) and 1.42 is the factor for conversion to volume O_2 (Kenner and Ahmed, 1975).

CAT was determined according to Aebi (1984). A volume of 0.3 mL of homogenate was mixed with 1.7 mL of 50 mM (pH 7.0) potassium phosphate buffer. The reaction was started by adding 1 mL of 30 mM hydrogen peroxide, prepared in 50 mM potassium phosphate buffer pH 7.0, to obtain a hydrogen peroxide concentration of 10 mM. The decomposition of hydrogen peroxide was followed spectrophotometrically by measuring absorbance every 30 s at 240 nm for 2 min at 25 °C using quartz cuvettes with a path length of 10 mm and a Lambda UV/Vis spectrophotometer (PerkinElmer, Waltham, MA, USA). One U was defined as the amount of CAT that degrades one μmol of hydrogen peroxide in one minute (ε_{240} = 43.6 M^{-1} cm^{-1}). These results were divided by the total amount of protein and expressed as specific CAT activity per milligram of protein. The protein concentration of the homogenate was determined with a Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Absorbance of the samples was measured using a microplate reader (Synergy MX BioTek).

CAR was determined using a commercially available Protein Carbonyl Content Assay Kit (MAK094 Sigma-Aldrich). In this kit, after addition of 2,4-dinitrophenylhydrazine (DNPH) to the samples, stable dinitrophenyl (DNP)-hydrazone adducts are formed that are proportional to the carbonyls present and can be detected spectrophotometrically at 375 nm. Samples were homogenized in 300 μL of 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 1 mM PMSF. The homogenates were centrifuged at 13,000 ×*g* for 13 min in a refrigerated centrifuge, and the supernatant was analysed according to the manufacturer's instructions.

2.4. Q_{10} and E_a

The temperature coefficient (Q_{10}) represents the factor by which the rate of a reaction increases for every 10-degree rise in temperature (Bennett and Dawson, 1976). The Q_{10} values were calculated according to the equation in Lampert (1984):

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$$

where for the reaction rates R_1 and R_2 we used PMA values at the low (T_1) and high (T_2) thermal treatments, respectively.

Arrhenius activation energy (E_a) is a 'temperature characteristic' that describes the accelerating effect of temperature on metabolic rate. It is determined from the least square slope of an Arrhenius plot of the natural logarithm of metabolic rate or activity (in our case PMA) against the reciprocal of absolute temperature (Robinson and Williams, 1993) according to the following equation:

$$E_a = -R_{gc} \times S$$

where E_a is expressed in kJ/mol, R_{gc} is the gas constant (8.31 J mol^{-1} $degree^{-1}$), and S is the slope of the Arrhenius plot.

2.5. Statistical analyses

All statistical tests were performed in R studio 2023.06.1, R version 4.3.1 (R Core Team, 2020). The variables autocorrelation was first examined using a principal component analysis (PCA) without a Vari-Max rotation of the axes, which is used to analyse the contribution of all quantitative variables to the variability of the data (Jolliffe, 2002). To test for differences among species, we performed separate linear mixed models for HB, CAT, CAR and PMA, in which we included species as fixed effect, elevation as an ordinal random effect and BCI as continuous random effect. When analysing PMA, we also included the temperature at which PMA was measured as ordinal fixed factor in the model and the individual code of every lizard was treated as a random variable to correct for repeated measures. Body condition index (BCI) was included into the model as random variable.

We built the most complex model with BCI as random effect (and temperature in interaction with species or as separate fixed factor for PMA) and all less complex models to compare them. In all cases, the model with the lowest AICc was selected as the most informative model (Cavanaugh and Neath, 2019). Both marginal and conditional R-squared values were calculated for all models to explore the contribution of fixed and random factors to the proportion of total variance explained. We then created ANOVA tables for fixed effects of the most informative models to facilitate interpretation of the results. Homogeneity of variances (of model residuals) was tested with Levene's test and normality was tested with the Shapiro-Wilk test before performing the ANOVA analysis. To compare Q_{10} values among species we used an ANOVA followed by post-hoc tests (Tukey's HSD). Values of Q_{10} were log transformed prior to analysis to assure normality in the data. Post-hoc pairwise differences within significant categorical predictors included in the final models were calculated using the *multcomp* package (Hothorn et al., 2008) and the *emmeans* package (Lenth, 2019). Dataset is available in data repository under doi: https://doi.org/10.5281/zenodo.10256237.

3. Results

3.1. Data variability

PCA analysis reveals that the first dimension explained 46.1% and

the second dimension explained 17.9% of the variance observed (Appendix Fig. A). The highest contributions to the PC1 were PMA values measured at four different temperatures (Fig. 2A, Appendix Fig. A). For PC2, the highest contributions were of HB and CAR, (Fig. 2A, Appendix Fig. A). The variables corresponding to *I. monticola* (most phylogenetically distant species and sampled at the highest elevation of 1990 m a.s.l.) are positioned away from all other species (Fig. 2B). In contrast, two native species (*P. carbonelli* and *P. virescens*), both originating from low elevations (145 m a.s.l.), exhibit a big overlap in the PCA space (Fig. 2B). There is also a substantial overlap between the nonnative *P. siculus* and *P. lusitanicus*, both sampled at contrasting elevations (2 and 1400 m a.s.l., respectively, Fig. 2B).

3.2. Effects on metabolic traits

The models and the selection of models based on the AICc values are shown in Table 2. Correlations between measured parameters (HB, CAT, CAR and PMA) are mainly determined by species (and temperature in the case of PMA) and not by BCI, since the most informative models were always those that this factor (Table 2). In the case of HB, variation is only partially explained (27.5%) by species, while both fixed and random effects (species and elevation) together explain 54.3% of the variance (Table 2). The variation in CAT is mainly explained by species differences (41.8%) (Table 2). Variation in CAR follows a similar pattern to HB (species explained 31.7% and species and elevation together 59.9% of the variance, Table 2). Finally, both fixed and random effects explained 91.4% of the variance in PMA, suggesting that species together with incubation temperature and elevation correlate with PMA (Table 2).

Our linear mixed model results for HB showed the intercept is highly significant (DF = 1, Chi-square = 45.341, $p > 0.001$), which indicates that the baseline level of HB is significantly different from zero. The effect of species on HB is not statistically significant (DF = 4, Chi-square = 5.730, $p = 0.220$, Fig. 3A). In CAT, both the intercept (DF = 1, Chi-square = 32.038, $p < 0.001$) and the species effect (DF = 4, Chi-square = 34.007, $p < 0.001$) are significant. The species *P. virescens* shows a significant difference in catalase levels compared to *I. monticola* and *P. carbonelli* (Post-hoc Tukey test: $P = 0.022$ and $P \leq 0.001$, respectively, Fig. 3B). Other comparisons did not show significant differences at the

Table 2
Linear mixed models (LMM) built for four subcellular metabolic traits with AICc scores comparing the models. Selection of best-fitted model based on the lowest AICc value and ΔAICc above 2 compared with the other model(s). Best-fit models are shown in bold. HB = hemoglobin concentration, CAT = catalase activity, CAR = carbonyl concentration, PMA = potential metabolic activity, BCI = body condition index, Elev = elevation, Temperature = temperature regime of PMA, R^2m = marginal r square (proportion of variance explained by the fixed effects alone), R^2c = conditional r square (proportion of variance explained by both fixed and random effects).

	Models	R^2m	R^2c	AICc
HB	**HB ~ Species + (1|Elev)**	**0.275**	**0.543**	**208.2**
	HB ~ Species + (1 + BCI|Species) + (1|Elev)	0.167	0.723	217.2
CAT	**CAT ~ Species + (1|Elev)**	**0.418**	**0.490**	**260.5**
	CAT ~ Species + (1 + BCI|Species) + (1|Elev)	0.210	0.744	269.5
CAR	**CAR ~ Species + (1|Elev)**	**0.317**	**0.599**	**183.3**
	CAR ~ Species + (1 + BCI|Species) + (1|Elev)	0.200	0.746	192.3
PMA	**PMA ~ Species + Temperature + (1|Elev) + (1|ID)**	**0.331**	**0.914**	**15.7**
	PMA ~ Species*Temperature + (1|Elev) + (1|ID)	0.387	0.915	52.4
	PMA ~ Species + (1 + BCI|Species) + (1|Elev) + (1|ID)	0.050	0.882	197.7
	PMA ~ Species + (1|Elev) + (1|ID)	0.164	0.589	191.0

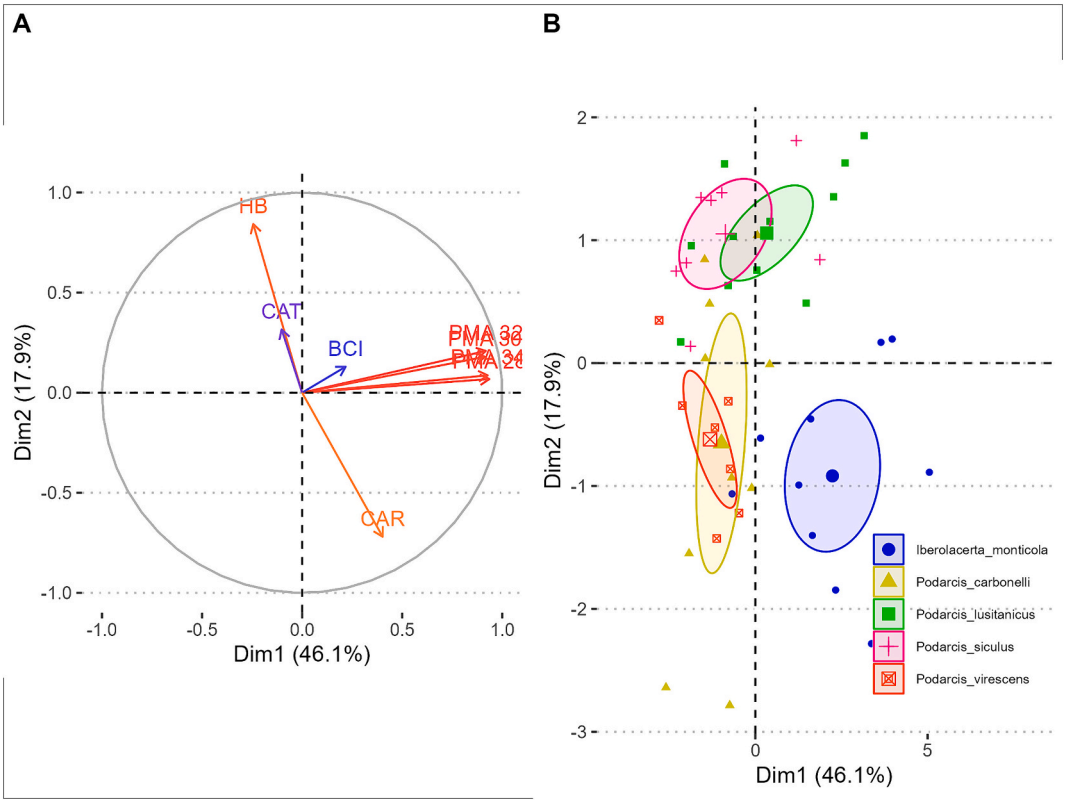

Fig. 2. Results of PCA analysis of all variables. The variable correlation plot (A) shows the relationship between all quantitative variables where positively correlated variables are grouped together and the distance between variables and the origin measures the quality of the variables on the factor map. Variables that are away from the origin are well represented on the factor map. The graph of individuals (B) plots individuals coloured by Species. Ellipses represent 95% confidence intervals.

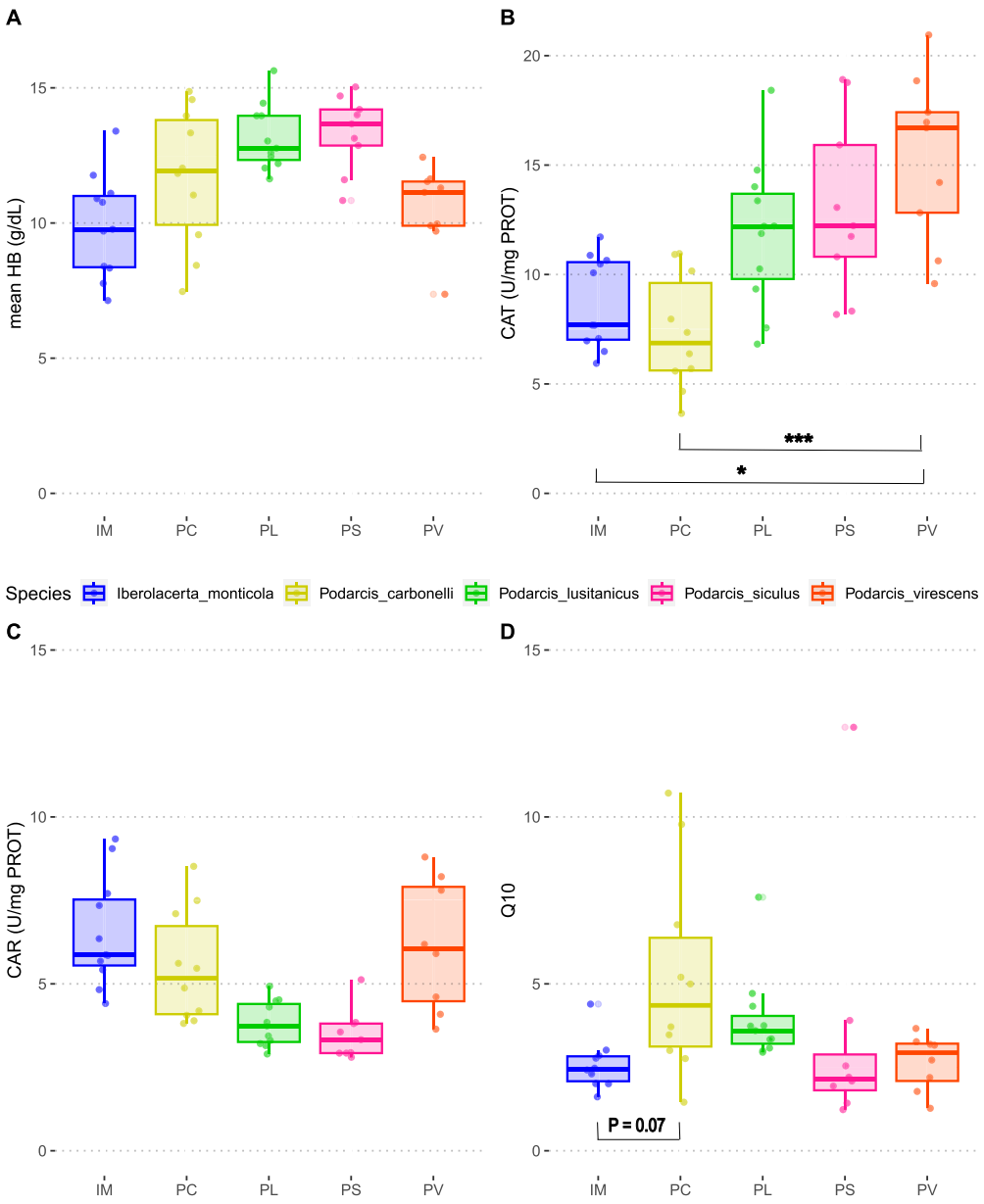

Fig. 3. Box plots of means, interquartile values, and minimum and maximum values of four subcellular metabolic traits measured in five lacertid species: (A) hemoglobin concentration (HB), (B) catalase activity (CAT)), (C) carbonyl concentration (CAR), and (D) temperature coefficient (Q_{10}). Significant pairwise comparisons of CAT between species are marked with black lines and asterisks corresponding to *P* values: * ≤0.05, ** ≤0.01, and *** = $P < 0.001$. IM = *Iberolacerta monticola*, PC = *Podarcis carbonelli*, PL = *P. lusitanicus*, PS = *P. siculus*, and PV = *P. virescens*.

0.05 level, except for *P. siculus* versus *P. carbonelli*, which is marginally significant (Fig. 3B). Similarly to HB, in CAR the intercept is significant (DF = 1, Chi-square = 27.002, p > 0.001) and the effect of species on CAR is not statistically significant (DF = 4, Chi-square = 5.181, p = 0.269) showing that there are no significant differences in the mean CAR levels among different species (Fig. 3C). In PMA, the intercept (DF = 1, Chi-square = 28.0611, p < 0.001) was significant, and the species effect was not significant (DF = 4, Chi-square = 1.2778, p = 0.865), while temperature was (DF = 3, Chi-square = 421.4560, p < 0.001). All temperature comparisons show highly significant differences in PMA: PMA levels at 30 °C are significantly higher than at 28 °C, PMA levels at 32 °C are significantly higher than at 28 °C and 30 °C and PMA levels at 34 °C are significantly higher than at 28 °C, 30 °C, and 32 °C (Fig. 4). (See Table 3).

3.3. Rates of metabolic respiration across species

The mean Q_{10} significantly differ among the species (DF = 4, F = 3.108, P = 0.025). However, the pairwise comparisons show no significant differences between the species pairs, although the comparison of *P. carbonelli* and *I. monticola* was close to the significance level of 0.05 (P = 0.071). The Q_{10} calculated for *I. monticola* was the lowest among the five species (Table 4, Fig. 3D) indicating that the rate of increase in metabolic respiration with increasing temperature is the lowest in this species. Also, a magnitude of response to small temperature changes (E_a) calculated over the tested temperature range (28–34 °C) was the lowest for *I. monticola*, followed by *P. virescens*, and *P. siculus*, whereas *P. lusitanicus* and *P. carbonelli* had relatively high E_a values (Table 4).

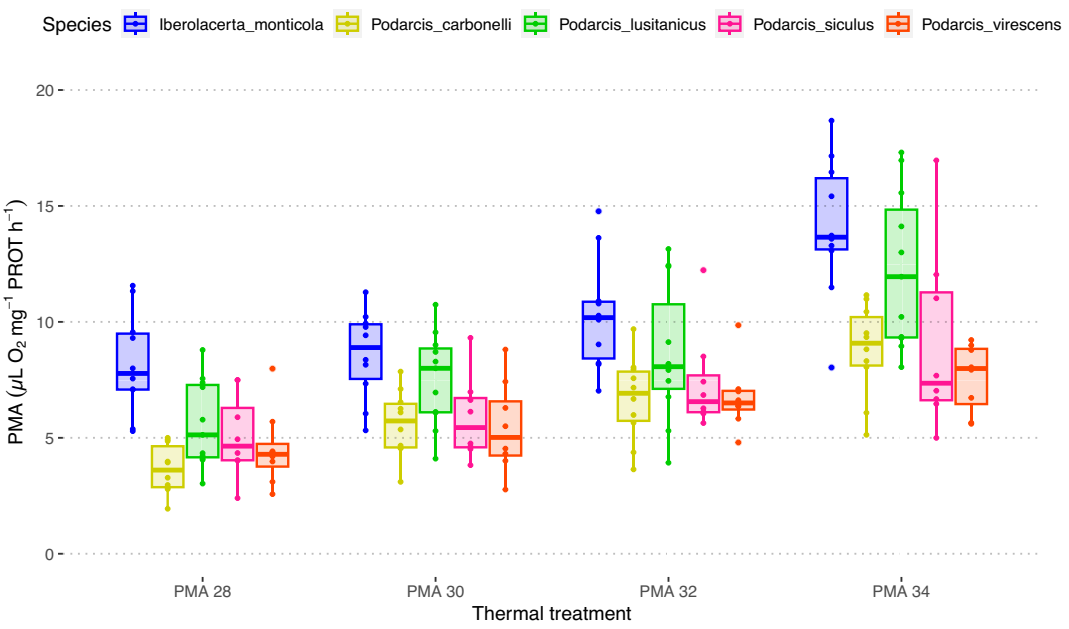

Fig. 4. Box plots of means, interquartile values, and minimum and maximum values of the potential metabolic activity (PMA). PMA was measured at four different temperatures (28, 30, 32 and 34 °C), which are indicated on the x-axis. IM = *Iberolacerta monticola*, PC = *Podarcis carbonelli*, PL = *P. lusitanicus*, PS = *P. siculus*, and PV = *P. virescens*.

Table 3
ANOVA tables of best-fit linear models for four subcellular metabolic traits. HB = hemoglobin concentration, CAT = catalase activity, CAR = carbonyl concentration, PMA = potential metabolic activity, Temperature = temperature regime of PMA, DF = degrees of freedom, Chi sq. = Wald chi square value, and P = *P* value. Statistically significant predictors with $P \leq 0.05$ are shown in bold. The ":" indicates an interaction effect between predictors.

	Predictor	DF	Chi-sq	P
HB	**Intercept**	**1**	**45.34**	**<0.001**
	Species	4	5.73	0.220
CAT	**Intercept**	**1**	**32.04**	**<0.001**
	Species	**4**	**34.01**	**<0.001**
CAR	**Intercept**	**1**	**27.00**	**<0.001**
	Species	4	5.18	0.269
PMA	**Intercept**	**1**	**28.06**	**<0.001**
	Species	4	1.28	0.865
	Temperature	**3**	**421.46**	**<0.001**

Table 4
The mean rate of metabolic respiration at temperatures differing by 10 units (K) (Q_{10}) with standard error and the Arrhenius activation energy (E_a) values calculated from the potential metabolic activity (PMA) values in the range of body temperatures between 28 and 34 °C.

Species	mean Q_{10}	SE Q_{10}	E_a (kJ/mol)
Podarcis virescens	2,65	0,29	73,56
Podarcis siculus	3,50	1,34	74,84
Podarcis carbonelli	5,19	0,97	109,31
Podarcis lusitanicus	3,95	0,40	96,46
Iberolacerta monticola	2,59	0,24	70,06

4. Discussion

Subcellular level traits related with metabolism that are involved in oxygen delivery, activity of the electron transport chain and the antioxidant activity were compared among five different species of Lacertid lizards in our study. The species *I. monticola* which is a mountain specialist and is most distantly related to the other species occupied a comprehensively divergent place in the physiological space. It showed the lowest temperature response of metabolic rates and one of the lowest values of catalase activity. It is, however, important to acknowledge that the species in our study do not represent statistically independent observations due to their phylogenetic relationship. Using the Phylogenetic Independent Contrast method (PICs, Felsenstein, 1985; Garland Jr. et al., 1992), we could test for differences in traits between species to determine weather the observed changes were caused by natural selection. Unfortunately, this was not possible with the limited number of species (five) studied here. The species were sampled at an altitudinal range of almost 2000 m; therefore, elevation was included as a random factor. Elevation helped to explain a greater proportion of variance in the hemoglobin level, carbonyl concentration, and rates of potential metabolic activity, but not in catalase activity, where we found significant species differences. Finally, it is known that body condition may have an effect on metabolic traits (Brown et al., 2004), but we found no significant effect of body condition index on any of the physiological traits studied.

Hemoglobin levels can vary significantly across species and with environmental factors (Hochachka and Somero, 2002). Our results show no significant interspecific differences in hemoglobin concentration. However, in combination with elevation, which was included as a random factor, about 50% of the hemoglobin variation could be explained. Contrary to the expectation that at high altitudes, where oxygen partial pressure is lower, hemoglobin concentration increases to enhance oxygen carrying capacity, we found that the species sampled at the sea level, *P. siculus*, had the highest hemoglobin concentration. On the other hand, *P. lusitanicus* sampled at 1400 m above sea level also had high a hemoglobin concentration, as expected, while the other species had an intermediate concentration. It seems that the hemoglobin concentration exhibits species-specific and not altitude-specific differences.

The species differed in their catalase activity, but only *P. virescens* had increased catalase production compared to *I. monticola* and *P. carbonelli*. Catalase is responsible for attenuating the harmful effects of ROS (Suzuki et al., 2010), thus, high levels in *P. virescens* indicate that this species has increased antioxidant activity. Moreover, elevated carbonyl protein levels indicate that the proteins were oxidatively modified, indicating increased oxidative stress. High carbonyl protein levels were also observed in *P. virescens* to be high. High catalase activity combined with high carbonyl protein content indicates a state of

increased oxidative stress, where the cell shows a strong antioxidant response but is still exposed to significant oxidative damage, which could indicate an adaptive response to chronic oxidative stress (e.g. Lushchak, 2011). In contrast, the lowest levels of catalase activity were observed in *P. carbonelli* and *I. monticola*. The lower catalase activity in these species suggests that they may be less able to neutralize hydrogen peroxide (Suzuki et al., 2010), which could make them more susceptible to oxidative stress compared to other species in the study. Both species also had relatively high levels of carbonyl proteins (suggesting increased oxidative damage to proteins), but further investigation would be required to understand whether high ROS production causes high oxidative stress in this species.).

Potential metabolic activity, which indicates the maximum rate at which enzymatic reactions of aerobic respiration can occur in the cell (Muskó et al., 1995), did not differ significantly among the five species, but the temperature between 28 and 34 °C at which we measured PMA had a significant positive effect on metabolic activity for all species. This temperature range corresponds to the preferred body temperatures of the study species, and their metabolic rates respond positively to temperature changes in this interval, as previously found in other lacertid species (e.g. Žagar et al., 2018a; Serén et al., 2023). We also calculated the rate of change of metabolic rate with temperature (encapsulated in Q_{10}). The calculated Q_{10} for *I. monticola* was the lowest among the five lizard species. This suggests that the metabolic rate of *I. monticola* is less sensitive to temperature fluctuations, which could be beneficial in stable high-mountain environments where temperature fluctuations are more moderate or less frequent than in the lowlands.

Several species of the genus *Iberolacerta*, including *I. monticola*, are all distributed in mountains and can efficiently thermoregulate despite varying environmental temperatures and operate at a higher preferred body temperature compared to *Podarcis* species (e.g. Osojnik et al., 2013, but see also Aguado and Braña, 2014; Sannolo and Carretero, 2019; Garcia-Porta et al., 2019). Low environmental temperatures shorten the duration of activity periods of lizards at high altitudes, and this could be the selection pressure for high-latitude species to have a higher thermoregulatory set-point and more precise thermoregulation (Ortega et al., 2016; Žagar et al., 2018a). Moreover, lower Q_{10} value, as found in our study could indicate efficient energy utilization over a range of temperatures, supporting the precise thermoregulation known for this high-altitude species. *Iberolacerta monticola* also had low activation energy (E_a values), indicating that little energy is required for metabolic reactions to occur (Brown et al., 2004) in this species. This can be advantageous in environments where energy conservation is critical, such as mountains with colder temperatures and lower productivity (i.e. fewer food resources (Körner, 2007)), as metabolic reactions can proceed more easily with lower energy costs. On the other hand, *Podarcis* species tend to be more generalist and have wider distribution ranges also in relation to altitude (Sillero et al., 2014). In our study, some *Podarcis* species had higher and some lower values of Q_{10} and E_a, but these differences were not significant. Thus, the observed differences could be due to random variation rather than actual biological differences. The lack of significant differences may suggest that their metabolic responses to temperature are broadly similar, allowing them to thrive in a range of environmental conditions across different altitudes but further research using larger sample sizes and multiple populations would give us a better understanding.

In summary, we have found species differences in the catalase activity among five lacertid species. Differences in HB, CAR and PMA could not be attributed to species, but together with elevation approximately half of variance was explained. Temperature had a positive and parallel effect on PMA across all species. It should be noted that we only sampled one population per species and were unable to capture adaptations in ecophysiology to species range (as suggested for example by Carretero and Sillero, 2016 for *P. carbonelli*). This should be taken into account when interpreting the results – thus ecophysiology reflects not only the local adaptation of the sampled populations but possibly also the evolutionary history of the species.

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CRediT authorship contribution statement

Anamarija Žagar: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Urban Dajčman:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis, Data curation. **Rodrigo Megía-Palma:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Tatjana Simčič:** Writing – review & editing, Supervision, Methodology, Data curation. **Frederico M. Barroso:** Writing – review & editing, Methodology. **Senka Baškiera:** Writing – review & editing, Methodology. **Miguel A. Carretero:** Writing – review & editing, Supervision, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Dataset is available in data repository under doi: 10.5281/zenodo.10256237

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111729.

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