









# Functional responses in a lizard along a 3.5-km altitudinal gradient

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## Abstract

**Aim:** Physiological and metabolic performance are key mediators of the functional response of species to environmental change. Few environments offer such a multifaceted array of stressors as high-altitude habitats, which differ markedly in temperature, water availability, UV radiation and oxygen pressure compared to low-altitude habitats. Species that inhabit large altitudinal gradients are thus excellent models to study how organisms respond to environmental variation.

**Location:** Tenerife island, Canary Islands archipelago (Spain).

**Taxon:** Tenerife lizard (*Gallotia galloti*, Lacertidae).

**Methods:** We integrated data on age structure, thermal and hydric regulatory behaviour and four metabolic and stress-related biomarkers for an insular lizard that inhabits an extreme altitudinal range (sea level to 3700 m a.s.l.), to understand how ectotherms' age, ecophysiology and metabolism can be affected by extreme environmental variation.

**Results:** We found marked differences in metabolic stress markers associated with altitude (particularly in the abundance of carbonyl metabolites and relative telomere length), but without a linear pattern along the altitudinal cline. Contrary to expectations, longer telomeres and lower carbonyl content were detected at the highest altitude, suggesting reduced stress in these populations. Evaporative water loss differed between populations but did not follow a linear altitudinal gradient. Lizard age structure or thermal physiological performance did not markedly change across different altitudes. Mixed signals in life-history and thermal ecology across populations and altitude suggest complex responses to variable conditions across altitude in this species.

**Main Conclusions:** Our integrative study of multiple functional traits demonstrated that adaptation to highly divergent environmental conditions in this lizard is potentially linked to an interplay between plasticity and local adaptation variably associated with different functional traits.

## KEYWORDS

ecophysiology, evaporative water loss, metabolic activity, oxidative stress, preferred temperatures, relative telomere length, skeletochronology

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## 1 | INTRODUCTION

The impact of global change on biodiversity is a major conservation concern given the rapid pace of biodiversity loss (Ceballos et al., 2017; Huey et al., 2012). However, disentangling the roles of long-term adaptation and phenotypic plasticity in the response to environmental variation remains challenging. Multiple biotic and abiotic factors interact to generate these complex biological responses, and important recent advances to identify signals of these responses on the phenotype of several species provide important insights for the planning of realistic conservation strategies (Harmon & Pfennig, 2021; Mawdsley et al., 2009).

A promising strategy for understanding biological responses to environmental pressures is targeting model species that inhabit wide biological gradients. Altitudinal gradients, in particular, can act as a proxy for temporal shifts induced by climate change (Körner, 2007). Coping with the multitude of abiotic factors that vary in these contrasting habitats (e.g. oxygen pressure, temperature, relative humidity, ultraviolet radiation), often within short geographic ranges, requires a suite of behavioural and physiological responses such as optimization of respiration, thermoregulation, physiology or gut microbiota (González-Morales et al., 2021; Howald & Hoppeler, 2003; Quagliariello et al., 2019; Storz & Scott, 2019; Žagar et al., 2018, 2022).

In this context, ectothermic vertebrates are key models in environmental change studies due to their strong dependency on the surrounding environment to regulate body temperature (Angilletta, 2009; Huey, 1982; Sinervo et al., 2010), which should make them more vulnerable to environmental change (specifically climate change) than endotherms (Paaijmans et al., 2013). Inversely, several studies suggest that ectothermic vertebrates can be fairly resilient to environmental change, especially if they have a pronounced plastic ability to respond to such changes (which is often the case, see Crowell et al., 2021 and Seebacher et al., 2015). Lizards in particular are useful for this type of research since most species have a relatively limited home range and have short generation times, which makes them especially susceptible to environmental change (Pianka & Vitt, 2003). Moreover, many species are suitable for experimental manipulation, since they are easily captured and cope well with captivity in laboratory facilities (Pianka & Vitt, 2003).

Although lizards live under a wide range of thermal conditions, a recent study by Garcia-Porta et al. (2019) encompassing over 200 species of lacertids showed a partial correlation between hydroregulation and climate, while thermal physiology remained phylogenetically conservative. This reinforces earlier suggestions that lizards, particularly in temperate areas, may be susceptible to ongoing global warming (but see Camacho et al., 2021). Environmental stress associated with climatic and altitudinal shifts may require lizards to change their thermal and hydric regulatory responses to maintain fitness and performance (Angilletta et al., 2002; Belasen et al., 2017; Cowles & Bogert, 1944; Hertz et al., 1979; Jiang et al., 2023). For example, studies linked dehydration in lizards with reduced

reproductive output and developmental rates (Lorenzon et al., 1999; Wang et al., 2016), which requires responses such as lowering the preferred body temperature or abandoning activity to prevent water loss (Sannolo & Carretero, 2019). The impact of altitude remains poorly understood, with only a single study demonstrating that lizard populations at higher altitudes are more resistant to water loss (Sannolo et al., 2020).

Other abiotic factors besides temperature (e.g. humidity, oxygen pressure, UV exposure) are also likely to influence metabolism of ectotherms since they can have impact on physicochemical processes (Rozen-Rechels et al., 2019; Rubalcaba et al., 2020). It has been shown also in lizards that the speed of metabolic response will depend on whether they show niche conservatism or plastic physiology (Ahmadzadeh et al., 2013; Gangloff et al., 2019; Megía-Palma, Jiménez-Robles, et al., 2020). Studies on metabolism and oxidative-stress markers can elucidate the physiological responses of lizards to altitude (e.g. Seebacher et al., 2012; Žagar et al., 2015; Žagar et al., 2018). For example, hypoxic environments at very high altitudes (>3000 m a.s.l.) are known to increase the production of reactive oxygen species and trigger cell oxidative stress reactions (Gaur et al., 2021), which induces higher activity rates of antioxidant mechanisms to buffer reactive oxygen species and prevent cell damage (Zamocky et al., 2008). Despite these indications of metabolic stress in high altitude, it is still unclear if this is reflected in lower lizard survival, which is a complex question due to many other confounding pressures (e.g. predation pressure) (Martín & López, 1996).

Regardless of animals' thermal regime, theory suggests that higher altitudes lead to a shift towards a slower life-history strategy (reviewed in Laiolo & Obeso, 2017), which is often attributed to longer developmental periods, higher body mass and longer lifespan (Boyle et al., 2016; Western & Ssemakula, 1982). Environmental factors seem to be the main drivers of this trend, namely thermal amplitude, low temperatures, shorter activity seasons, lower food availability, lower climate predictability and lower predation risk (Laiolo & Obeso, 2017; Sandercock et al., 2005; Turbill et al., 2011; but see Sears, 2005). For squamates, no clear trends in age structure have been found associated with altitude (Cabezas-Cartes et al., 2018). Interestingly, two recent studies involving age structure data from altitudinal gradients have found shorter lifespan in medium altitudes comparing with the extremes of the studied gradient (Altunışık et al., 2022; Comas et al., 2020). Many of these contrasting results highlight our limited understanding about species adaptations to these extreme environments.

Another widely used biomarker of oxidative stress is the telomere length of somatic cells, which is not only affected by stressors such as UV, immune challenges and thermal stress (Epel et al., 2004; Friesen et al., 2022; Rochette & Brash, 2010) but also by age (Whittemore et al., 2019). In lizards, studies using telomere lengths are scarce and converge to a complex association of telomere size with altitude where despite the higher potential for reactive oxidative stress, the association between telomere size and altitude was either not evident (Burraco et al., 2020) or even positive (Fitzpatrick et al., 2021).

In this study, we focused on the life-history and ecophysiological responses to altitude in a lizard species, *Gallotia galloti* (Figure 1a). This species is endemic to the islands of La Palma and Tenerife, in the Macaronesian archipelago of the Canary Islands. On Tenerife, this species inhabits a wide range of altitudes, from sea level up to the summit of the Teide volcano at 3718 m a.s.l, experiencing this extreme altitudinal gradient within approximately 30 km (Figure 1b,c). The island setting provides advantages for studying environmental adaptation, because it reduces complexity of environmental conditions and due to lower interspecific competition (Jiménez-Robles & de la Riva, 2019). Currently, only four species of reptiles are present on this island, none of which represents a potential competitor to *G. galloti*, which is also the only reptile species at mid and high-altitudes (Pleguezuelos et al., 2002). A phylogeographic structuring between the North and South *G. galloti* populations is well documented

since clear phenotypic and genetic differences were described (Bischoff, 1982; Brown et al., 2006, 2016; Thorpe & Richard, 2001). Since only the southern lineage reaches the highest altitudes, we focused our sampling in the South.

To assess how *G. galloti* responds to an extreme altitude gradient, we collected data on the age, thermal and hydric physiological traits, as well as biomarkers of metabolic activity and stress from lizards living at different altitudes. We hypothesize that physiological stress associated with restrictive environmental conditions at the extreme of the altitudinal gradient may impose variable responses in life-history traits of *G. galloti*. We will test if populations from the harsher high-altitude environments will: (1) shift age structure (Laiolo & Obeso, 2017); (2) select for different preferred body temperatures (Meiri et al., 2013; Sannolo et al., 2020); (3) adjust water loss to humidity conditions (highest humidity at mid-altitude); (4) exhibit

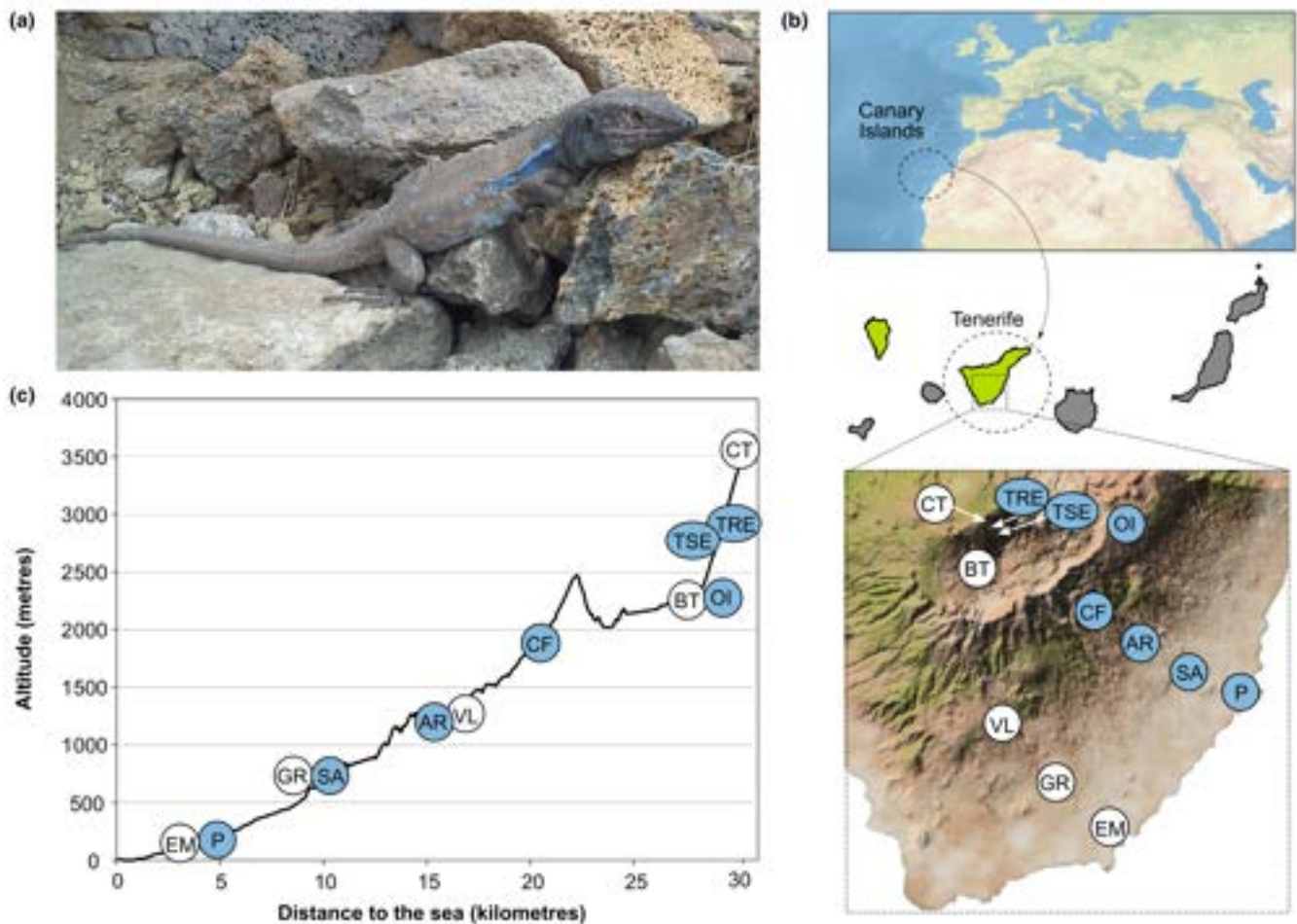


FIGURE 1 Altitudinal sampling design to study ecophysiology, metabolism, relative telomere length and age estimation through skeletochronology in an insular lizard. (a) The study species, *Gallotia galloti*, adult male. (b) Geographical location of the island of Tenerife in the Canary Islands archipelago (above, map by naturalearthdata.com), with a magnification showing the species' distribution (in the islands coloured in light green), as well as the sampling sites in Tenerife (below) with the following legend: CT—Cone of Teide (3600 m); TRE—High Teide Refugia (2900 m); TSE—Low Teide Refugia (2750 m); OI—Observatorio Izaña (2350 m); BT—Base of Teide (2300 m); CF—Corona Forestal (1900 m); VL—Villaflores (1350 m); SA—Sabinita (600 m); GR—Granadilla (600 m); P—Poris de Abona (150 m); EM—El Médano (100 m). White sampling sites have sample data for preferred temperatures, evaporative water loss experiments, potential metabolic activity, catalase, carbonyl, relative telomere length and age; blue sampling sites only have age data. (c) Location of sampling sites along a topographic profile on the southern side of Tenerife from the sea to the highest point of the island (following the location of the southeast sampling direction of the altitudinal transect).

differences in antioxidant enzyme activity due to higher reactive oxygen species production under high UV (Žagar et al., 2018); and (5) have different telomere lengths due to potential stress at higher altitudes (Bateson, 2016).

## 2 | MATERIALS AND METHODS

Fully detailed and reference methods are available in Appendix S1 in Supporting Information.

### 2.1 | Sampling

Samples of *G. galloti* on Tenerife were collected in two field expeditions held in July–August 2017 and 2018. Both sampling periods were climatically very similar according to the climate data of Tenerife (<https://weatherspark.com>). The sampling strategy implemented in 2017 was an altitudinal transect where we collected samples for age estimation, ecophysiological experiments, metabolic activity and relative telomere length whereas the 2018 sampling comprised replicated and additional altitudinal locations where we collected samples only for age estimation (Figure 1a, Table 1). Sampling was performed at an average of 630-m intervals from the sea level up to an altitude of approximately 3500 m a.s.l. (Figure 1b, Table 1). We confined our sampling to males to avoid the effects of reproductive cycle on ecophysiology, which are more intense and asynchronous in females due to vitellogenesis and pregnancy (e.g. Carretero, 2006). The sampling effort provided between seven and 19 lizards per location (Table 1). For each individual, we recorded the snout–vent length (SVL) to the nearest 0.01 mm with digital callipers and the initial body mass ( $M_0$ ) to the nearest 0.0001 g with a digital scale immediately before experimental procedures. After experiments, we collected one digit and blood samples.

### 2.2 | Skeletochronological analysis

To assess the age of the sampled males, we applied a phalangeal skeletochronology methodology (Comas et al., 2016; Guarino et al., 2010), clipping from each individual the fourth digit of one forelimb. This enabled us to determine lines of arrested growth (LAGs) in periosteal bone (Figure 2a). The number of LAGs, which correspond to the number of years (Castanet et al., 1993; Guarino et al., 2020), was counted by two independent observers (NS, 3 times and FMG, 2 times). We considered for analyses the final scores that were concordant or that diverged at maximum 1 year between the two observers and when this divergence occurred, the score from the most experienced observer (FMG) was preferred. Samples with more than 1-year divergence between different observers, dubious sections due to apparent bone rearrangement, very close LAGs or technical issues were not considered. For some samples, the second phalanx for the same individual was used if available. Since

we had phalanx sections of nearly hatched *G. galloti* provided by the museum collection of Koenig, we could apply the back-calculation method (Castanet et al., 1993; Guarino et al., 2008) to add LAGs completely removed by endosteal resorption to the actual number formed by the individual.

### 2.3 | Thermoregulation experiments

Experiments to determine preferred body temperature ( $T_p$ ) were performed with linear thermal gradients following the thermal gradient 10h method described in Koziel et al. (2021) (Figure 3a). Terraria with thermal gradients were built with PVC opaque walls with 100×30×40 cm and sandy soil from surrounding locations was used as substrate (~1 cm deep) with no shelter, food or water provided. A 150W infrared lamp suspended above one end of the terraria was used as heat source, generating a thermal gradient ranging between approximately 19°C and 68°C. Cloacal temperature measurements were performed every hour for 10 consecutive times (8:00–17:00, WEST time zone) using a type-k thermocouple HIBOK® 18 (precision 0.1°C) inside a 1.5-mm diameter probe to be inserted in the cloaca. Lizards from different localities were tested in randomized order within a period of up to 6 days.

### 2.4 | Evaporative water loss experiments

Experiments of evaporative water loss (EWL) were performed the day after the preferred body temperature assays (Figure 3b). In the intervening night, each lizard was placed inside a closed circular box with 10 cm Ø and 6 cm height containing small ventilation holes and a false bottom containing 5 grams of silica gel. Individual containers were moved to a larger opaque sealed chamber (48×38×30 cm) under constant humidity conditions (~25%) maintained with silica gel (following the protocol of Ferreira et al., 2016). At 7:00, each individual box without the false bottom was weighed using a digital balance (precision 0.0001 g) with measurements taken hourly for 12 consecutive hours (7:00–18:00).

Accumulated water loss ( $EWL_a$ ), which provides a measurement of water lost by lizards throughout the experiment, was calculated using an initial body mass measurement recorded before the start of the experiment ( $M_0$ ) and using hourly records of mass ( $M_n$ , first measurement at 8:00 not considered). The applied formula was:  $EWL_a = [M_0 - M_n] / M_0$ .

### 2.5 | Metabolic activity assays

To test for potential metabolic activity (PMA), we cut approximately 10 mm length from flash frozen tail tip tissue and homogenized it with a mortar and liquid nitrogen. The moist homogenized tissue was then weighed and prepared for incubation. Incubation was carried out in parallel at three different incubator chambers



TABLE 1 Number of individuals from each sampling site in relation to the analysed variables.

Altitude (ma.s.l.)	Location	SVL (n)	Mass (n)	Age (n)	$T_p$ (n)	EWL (n)	PMA (n)	Catalase (n)	Carbonyl (n)	RTL (n)
100	EM	19	17	15	18	18	15	15	15	15
150	P	14	14	12	-	-	-	-	-	-
600	GR	19	19	17	18	18	17	17	17	19
600	SA	12	12	10	-	-	-	-	-	-
1300	AR	15	15	12	-	-	-	-	-	-
1350	VL	16	16	12	17	17	17	17	17	17
1900	CF	7	-	8	-	-	-	-	-	-
2300	BT	15	15	9	15	15	13	15	13	15
2350	OI	20	20	14	-	-	-	-	-	-
2750	TSE	-	-	8	-	-	-	-	-	-
2900	TRE	-	-	9	-	-	-	-	-	-
3550	CT	12	9	11	7	7	7	7	7	13
	TOTAL	149	137	137	75	75	69	71	69	79

Abbreviations: EWL, evaporative water loss; PMA, potential metabolic activity; RTL, relative telomere length;  $T_p$ , preferred temperature.

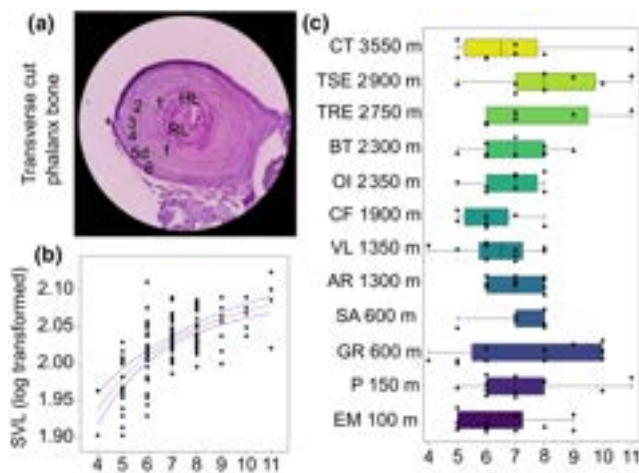


FIGURE 2 Representation of age estimation method and results (a) Example of age estimation based on lines of arrested growth (LAGs) in the phalanx of one analysed sample of *G. galloti*. HL, hatching line; RL, reversal line; f, false LAG; 5d, 5th counted LAG-year, which is very close to the previous LAG and seems like a double line (top left). (b) Scatterplot comparing skeletochronological age estimation with (log) SVL—snout-vent length (red line represents the *Michaelis-Menten* model, with the blue lines indicating the 95% confidence intervals). (c) Box-and-jitter plots indicating variation in age per population along the altitudinal gradient (altitude of each location is indicated).

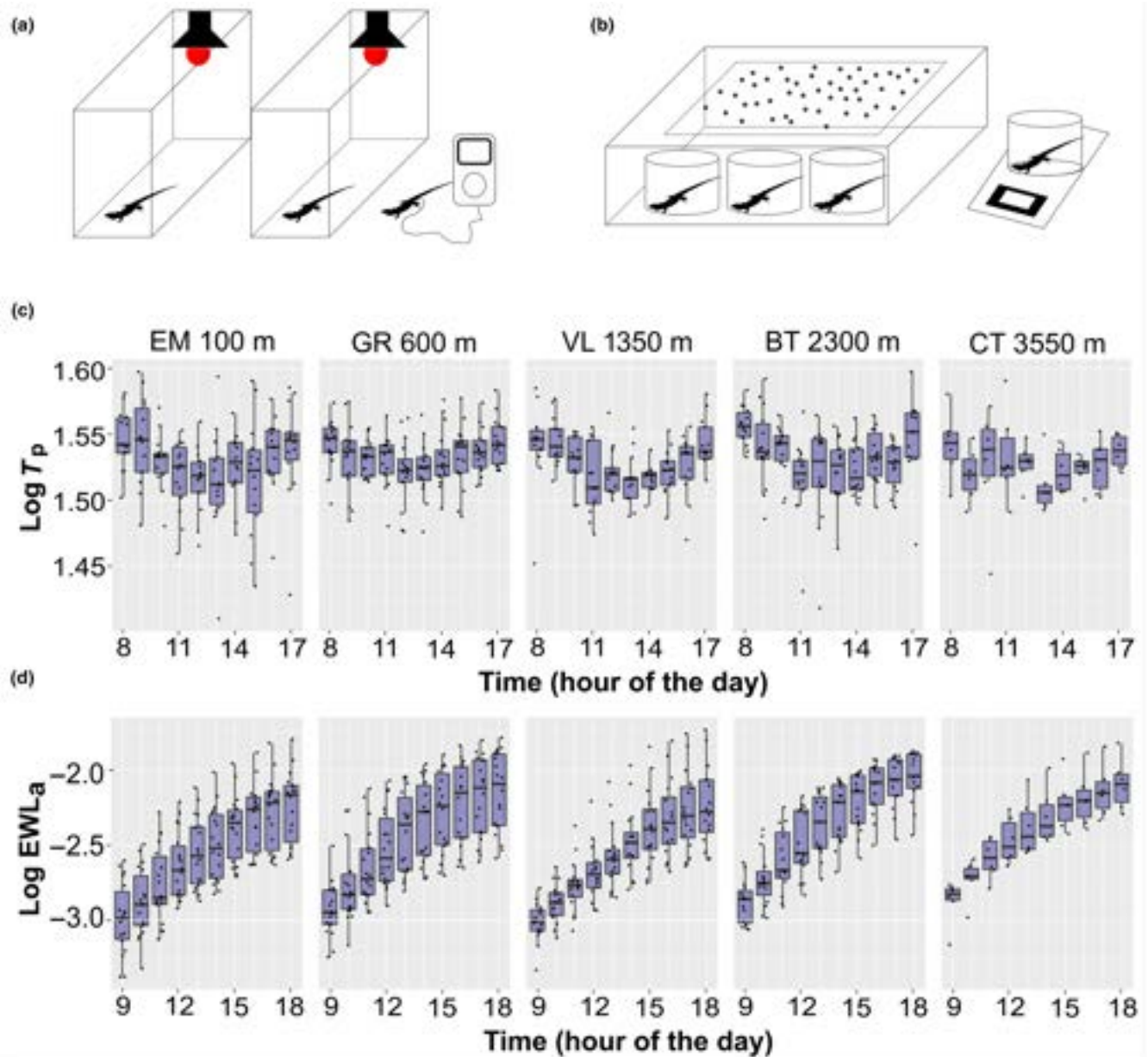
during 20min with three different temperature regimes (29°C, 33°C and 37°C) around the mean preferred body temperature, which in *G. galloti* is  $T_p=33.84^\circ\text{C}$  (see Appendix S1, Table S1.1). The values of PMA were estimated as the INT reduction capacity ( $\mu\text{LO}_2\text{mg}^{-1}\text{proteinh}^{-1}$ ) following the formula: INT reduction capacity =  $(\text{ABS}_{490} \times \text{Vr} \times \text{Vh} \times 60 \times 1.30) / (\text{Va} \times \text{S} \times t \times 1.42)$ ; where  $\text{ABS}_{490}$  is the absorption spectrum of the sample at a wavelength of 490nm; Vr is the final volume of the reaction mixture (mL); Vh is the volume

of the original homogenate (mL); Va 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  $\text{O}_2$  (Kenner & Ahmed, 1975). Catalase activity was assessed following the method of Aebi (1984). We expressed enzyme activities in enzyme units (U), in which one U is the amount of catalase that degrades one  $\mu\text{mol}$  of hydrogen peroxide in 1min. We divided these results by the total amount of protein to give the specific catalase activity per mg protein.

Carbonyl protein content was determined using a commercially available protein carbonyl content assay kit (MAK094 Sigma-Aldrich). In this kit, carbonyl content is detected spectrophotometrically at 375nm by derivatizing protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of stable dinitrophenyl (DNP)-hydrazone adducts proportional to the carbonyls present.

## 2.6 | Relative telomere length

To quantify the relative telomere length (RTL), we used the quantitative polymerase chain reaction (qPCR) method as described by Cawthon (2002), which compares the abundance of telomeric motifs to the abundance of a reference single-copy gene. Following the recommendations of Dagnall et al. (2017), analysis was restricted to blood samples collected within a short temporal period (within 15 days) and stored in the same manner (96% ethanol, at 4°C). The selected primers for telomere amplification were Tel1 (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2 (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') from Callicott and Womack (2006), which use a modified version from the original designed primers by Cawthon (2002). As a reference



**FIGURE 3** A schematic representation of experimental set-ups for studying thermal preferences and evaporative water loss, and results of both experiments. (a) For experiments on thermal preferences, lizards were placed in terraria heated with an infrared lightbulb at one end, allowing them to choose the distance to the heat source to optimize their thermoregulation; on the right: schematic procedure of cloacal temperature hourly measurement. (b) Evaporative water loss experiments required a box with silica to control air humidity and several small containers with the lizards inside; on the right: schematic procedure of the hourly mass measuring of lizards in a high-precision scale to account losses of mass due to water loss. (c) Location profiles of thermal preferences ( $T_p$ ) along the different periods of the day. (d) Location profiles of accumulated water loss (EWL<sub>a</sub>) along the different periods of the day. In each graph, populations are arranged (left-to-right) from the lowest altitude to the highest altitude.

single-copy gene, we amplified a fragment from the 18S gene, which primers had already been designed in another lacertid for qPCR (Andrade et al., 2019). The primers used for the amplification of the 18S gene fragment were 18SF (5'-AAACGGCTACCACATCCAAG-3') and 18SR (5'-CTCGATCCCAAGATCCAAG-3'). To further validate the suitability of this fragment as a reference, we verified the melt curve pattern, which presented a single peak. This indicates a single product amplification given that the melting temperature is sequence

dependent. In addition, a small random set of these amplification products were run on 2% agarose gel to check the single band indicative of a single product amplification. Three qPCR replicates were performed for each sample, and quantitation cycle values (C<sub>q</sub>) were averaged per sample/fragment to obtain the final value. To control plate efficiencies and intraplate variation, a calibrator sample was included on each plate of 96 samples. All plate calibrator efficiencies as calculated by Rasmussen (2001) fell within the acceptable range

with efficiency for the telomere assay of 97.4% and for the 18S assay of 93.7% (see Appendix S1, Table S1.2). To obtain a final measurement of RTL, we followed the formula of Pfaffl (2001), using the Cq values of the telomere and reference genes to calculate a ratio between the abundance of the telomeric motifs and the abundance of the reference single copy gene, taking into account the values of the calibrator samples.

## 2.7 | Statistical analyses

All analyses were conducted in R v4.2 (R Core Team, 2022), unless otherwise noted. Significance levels across the different analyses were considered at a threshold of  $p < 0.05$ . We began the analyses to verify basic morphometric relations, performing one-way analysis of variance (one-way ANOVA) (`aov` function in R) using SVL and mass separately as a proxy of size as a dependent variable and location as an independent variable. Robustness (body mass relative to length) across different locations was assessed with ANCOVA using mass as dependent variable and SVL as predictor (following the procedure of Ferreira et al., 2016).

After these analyses, allometric effects of body size on all numerical values of biochemical and physiological variables were removed using Thorpe–Leonart's method (Leonart et al., 2000) as implemented in 'Past' v4.10 (Hammer et al., 2001), with individual measurements of SVL as a proxy of size. These standardized measures were then transformed using a log10 transformation. Numeric values were identified as extreme outliers for each data set using Tukey's method (if they were larger than the third quartile +  $3 \times$  Interquartile range or smaller than the first quartile -  $3 \times$  Interquartile range) ('`identify_outliers`' function in 'rstatix' v.0.7.0 package in R), and thus, a total of 24 outliers were removed from statistical analyses. Final sample sizes for analysed variables per location are in Appendix S2, Table S2. Normality was assessed using Shapiro–Wilk's tests; most variables did not significantly deviate from a normal distribution, and the exceptions were verified visually using quantile–quantile plots and assessed to have minimum deviations. Homoscedasticity was confirmed using Levene's test (most variables did not have significant deviations). Assumption of sphericity for variables with repeated measures was checked with Mauchly's tests (no significant deviations in any variable).

The main objective of our analyses was to check for significant differences in age, physiological and biochemical variables (dependent variables) between populations that were caught at different altitudes. Differences between populations in variables without repeated measurements were assessed using one-way analyses of variance: One-way ANOVA ('`aov`' function in R) for age or Welch's ANOVA ('`oneway.test`' function in R) for non-homoscedastic variables—carbonyl metabolites and catalase activity. For variables that were collected using time point measurements throughout the day (preferred temperatures, accumulated water loss, instantaneous water loss) or parallel temperature measurements (potential metabolic activity), we used two-way repeated measures ANOVA (2-way

RM-ANOVA, '`anova_test`' function in 'rstatix' package in R) with locations as between-subject factor and time point/temperature as a within-subject factor. Pairwise differences between groups using *t*-tests were calculated using the function '`pairwise.t.test`' in R, and for pairwise differences from the same variables tested in ANOVA and Welch ANOVA analyses, the *p* value was adjusted with Benjamini–Hochberg procedure to reduce the false discovery rate at  $p < 0.05$ . To test for the existence of linear or nonlinear relationships between variables used in the models, we applied the '`nonlinear`' function in 'Past', which implements and compares the fit of the data for a variety of models using partial least squares. For each test, we selected the model with the lowest value of the Akaike information criterion (AIC) and assessed its significance by checking if the 95% confidence intervals of the  $\beta$  parameter (slope) overlapped with the null expectation of zero, which corresponds to an absence of a correlation (for linear relationships, we also assessed significance by calculating *p*-values through least squares regressions).

## 3 | RESULTS

Fully detailed tables with outputs of all statistical analyses are available in Appendix S2.

### 3.1 | Body size comparison

Populations differed significantly in morphological traits—SVL, mass and robustness (mass accounting for SVL) ( $p < 0.001$ ; Table 2). There was no linear relationship of these traits with altitude, while the pairwise comparisons showed that the lowest sampled population (EM) has significantly lower body mass than all other, except one at mid altitude (VL), while SVL was the lowest in mid-altitude population (CF) (Appendix S2).

### 3.2 | Age structure along the altitudinal cline

Age estimation through counting of LAGs in phalangeal histological sections (Figure 2a) was performed on 119 lizards; histological sections from an additional 18 lizards were discarded due to bad quality sections that made LAG counting impossible. A high variation in LAGs deposition was encountered in *G. galloti*, with double lines and false lines as already documented by Castanet and Baez (1988, 1991), although this did not prevent age estimation in the majority of specimens. According to previous study on other ectotherm species (Avens et al., 2017; Leclair Jr et al., 2000), the attainment of sexual maturity was interpreted as the first evident decrease of LAG spacing in individuals often seen at around 3–5 LAG-years.

Skeletochronological age consensus of the 119 samples was achieved for 71.4% of the samples, with the remaining 28.6% with a 1-year difference between observers. This enabled to verify whether populations inhabiting different altitudes show differences

**TABLE 2** Results of different analyses of variance (ANOVA) to assess variation in body size, and ecophysiological, metabolic and life-history variables in relation to sampling location in *G. galloti*.

	Effect	Total <i>df</i>	DFn	DFd	<i>F</i>	Generalized eta squares/sum of squares	<i>p</i> value
One-way ANOVA							
SVL	LOC	164	11	153	9.287	–	<0.001
Mass	LOC	128	8	120	6.185	–	<0.001
Age	LOC	116	10	106	1.069	–	0.394
ANCOVA							
Robustness	MASS	1			263.521	0.058	<0.001
	LOC	5			3.275	0.004	<0.001
Welch ANOVA							
Catalase	LOC		4	25.27	1.397	–	0.264
Carbonyl	LOC		4	24.05	4.305	–	<0.01
RTL	LOC		4	33.72	3.685	–	0.014
Two-way repeated measures ANOVA							
$T_p$	LOC		4	58	0.515	0.010	0.725
	HOUR		6.84	396.71	9.557	0.106	<0.001
	LOC:HOUR		27.36	396.71	0.863	0.041	0.668
EWL <sub>a</sub>	LOC		4	68	2.547	0.110	0.047
	HOUR		1.97	133.89	461.486	0.540	<0.001
	LOC:HOUR		7.88	133.89	0.523	0.005	0.835
PMA	LOC		4	64	2.954	0.133	0.026
	TEMP		2	128	139.369	0.281	<0.001
	LOC:TEMP		8	128	0.348	0.004	0.945

Note: Showing generalized eta squares for repeated measures ANOVA, sum squares for ANCOVA and two-way ANOVA.

Abbreviations: DFd, *df* in the denominator; *df*, degrees of freedom; DFn, *df* in the numerator; EWL<sub>a</sub>, accumulated evaporative water loss; LOC, sampling location; PMA, potential metabolic activity; RTL, relative telomere length; SVL, snout-vent length;  $T_p$ , preferred temperature.

in age structure. Skeletochronology confirmed that only 1.7% ( $n=2$ ) of all presumed adults collected for this study (by the absence of striped coloration typical from youngsters, Salvador, 2015) were not yet sexually mature (ages 2 and 3 years) and these samples were removed from all subsequent analyses. Log-transformed SVL and age varied in a nonlinear manner, following a Michaelis–Menten growth curve ( $AIC_{\text{Michaelis-Menten}} = 4.23$ ;  $a = 2.17 \pm 0.03$  95% CI; Figure 2b and Appendix S2). Age structure was not significantly different among populations (Figure 2c, Table 2).

### 3.3 | Thermo- and hydroregulatory profiles across the altitude

The thermal preferences of *G. galloti* were significantly different among the daily time points (Table 2, Figure 3c), while all tested populations along the altitudinal cline showed the same thermoregulatory profile, both in terms of the mean  $T_p$  and hourly pattern (Table 2 and Figure 3c). Hourly variation of  $T_p$  had a U-shape tendency in all populations, suggesting that *G. galloti* select for higher temperatures in the beginning and at the end of a day and slightly down-regulate

their inner temperature in the middle of the day when the environmental temperatures are higher.

EWL<sub>a</sub> patterns differed among the time points, but also changed significantly among locations (Table 2). There was no clear pattern indicating differences in EWL<sub>a</sub> measurements between altitudinal extremes (low- vs. high-altitude populations, Appendix S1). Pairwise comparisons indicate that the main between-location differences are associated with differences between measurements of a mid-altitude population (VL) and a low-altitude population (EM) compared to other populations, as these two populations lose less water than the rest.

### 3.4 | Biochemical signals of metabolism and stress

PMA exhibited significant variation across test temperatures and locations (Table 2, Figure 4a). Pairwise comparisons show that the population GR (low-altitude population at ~600 m a.s.l.) was the main cause for this significant effect, as PMA was higher in this population compared to the two sampled populations at the highest altitudes (BT at ~2300 m and CT at ~3550 m a.s.l., see Appendix S2). Catalase



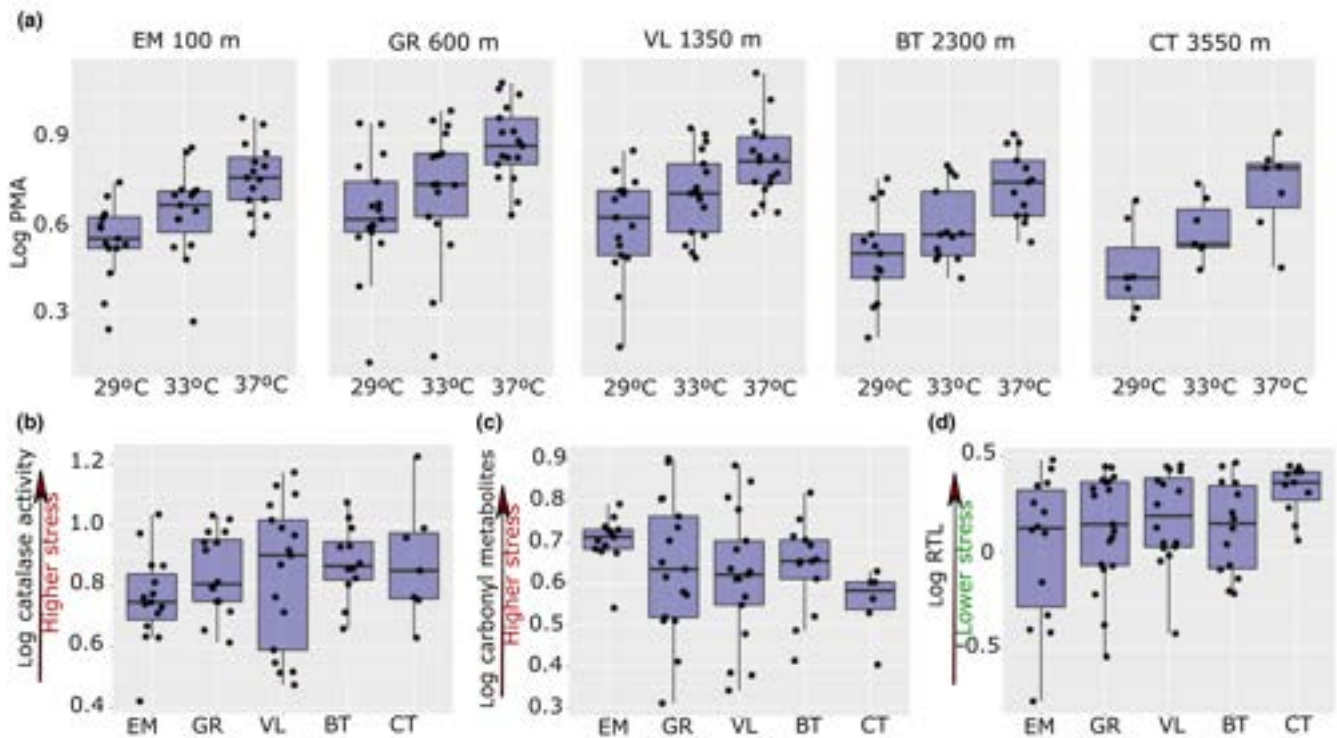


FIGURE 4 Box-and-jitter plots of studied physiological traits and relative telomere length (RTL). (a) Plots showing patterns of potential metabolic activity (PMA) in populations at different altitudes, with three testing temperatures (29°C, 33°C and 37°C). (b) Plots showing patterns of catalase activity in the different populations. (c) Plots showing patterns of carbonyl metabolite concentration in the different locations; (d) plots showing patterns of relative telomere length in the different locations.

activity levels did not vary between locations (Table 2, Figure 4b). Carbonyl protein content showed significant changes between populations (Table 2, Figure 4c), caused by differences between the locations at lowest (EM, highest value) and highest (CT, lowest value) altitudes (Appendix S2). RTL was significantly different between locations in the global test (Table 2, Figure 4d); when looking at the results of pairwise comparisons, none were significant after correction for multiple testing, but three contrasts (involving the highest altitude population, CT, against three lower altitude populations) were marginally significant ( $p=0.051$ , Appendix S2) and these represent a trend for longer RTL in the highest altitude population. We also found a significant linear inverse correlation between RTL and carbonyl content ( $AIC_{\text{linear}}=8.04$ ;  $p=0.01$ ) and non-significant relationships between RTL and age or SVL (Appendix S2).

## 4 | DISCUSSION

Understanding how ectotherms respond and eventually adapt to high altitudes is of great relevance in evolutionary biology, not only to better interpret how they thrive under conditions of increasing environmental harshness but also to predict how endangered species can respond to rapid global environmental and climate change. In this study, we show that different measures of performance of an ectotherm display variable patterns along an increasing altitude. Specifically, while we detected differences between lower and

higher altitude populations in stress-related biomarkers (carbonyl metabolites and telomere length), no changes in age structure were observed, indicating limited impact of stressors on individual survival. The detection of difference between populations in some ecophysiology traits (EWL<sub>a</sub> and PMA) but not in  $T_p$  suggests that colonization of high-altitude locations could be facilitated by the capacity to actively thermoregulate under different thermal condition, while metabolism suggests signs of local adaptation.

Despite the complex relationships between genetic adaptation and phenotypic plasticity that mediate how organisms cope with to environmental change (Corl et al., 2018; Ghalambor et al., 2007; Noble et al., 2019), it is increasingly recognized that this capacity of individuals to adjust their phenotype and behaviour based on external stimuli will be an important consideration for conservation efforts (Fox et al., 2019). Among the *G. galloti* populations sampled at different altitudes, the preferred temperatures were similar when lizards were placed in the same laboratorial conditions (no thermal restriction) and their body temperatures were recorded in a thermal gradient. This suggests that lizards from variable climatic conditions may need to devote different levels of their activity to thermoregulation to achieve optimal operating temperatures, or in very constrained habitats (either too hot and arid in the lowest altitude or too cold in the highest altitudes), they may not achieve their preferences and remain thermally suboptimal. Thus, temperature preferences are likely fixed and differences across elevations might contribute to keep it constant. A

similar finding has recently been reported in a smaller scale study using scattered populations of different subspecies of *G. galloti* in Tenerife (Albaladejo-Robles et al., 2022). The signal of potential plastic response in thermal ecology may partially explain the colonization success of this species at high altitudes, since such high-altitude environments can be thermally constraining to many other lizard species (Bodensteiner et al., 2021; Jiang et al., 2021; but see, e.g. Camacho et al., 2018 and Díaz de la Vega-Pérez et al., 2019 for similar scenarios to ours). Besides, there might be other advantageous conditions contributing to upward expansion, such as lower parasitization (Megía-Palma, Arregui, et al., 2020) and/or lower predation pressure near the volcano summit. Moreover, the highest populations do not seem to act as a sink population because the age structure of those populations does not differ from lowlands. However, it is important to note that although our approach of conducting thermal testing under standardized conditions at low elevation was necessary to understand how lizards from different altitudes respond to similar stimuli, this may also skew how we perceive organismal responses at the native altitude of different populations, particularly as different abiotic conditions at the test site may induce an abnormal response (e.g. see Gangloff et al., 2021). This highlights the need for further tests to support the plasticity hypothesis, in particular conducting translocation experiments to understand the capacity of individuals to adjust to changed environments.

In contrast to the absence of variation in thermal physiology, hydric physiology revealed differentiation among populations of the studied gradient. Water balance is an important feature of organisms as warmer and drier environments increase water vapour pressure deficit and cutaneous water loss (Anderson et al., 2022). Changes in precipitation within the altitudinal microclimates are known for the island and do not vary linearly with altitude (Juan et al., 2000), while they could have caused the observed variation between some of the populations due to local adaptation to humidity conditions. However, recent research of hydric physiology in lizards is revealing that water loss does not only reflect the physical properties of the skin, with epidermal lipids being the main barrier to water loss in lizards (Dmi'el, 2001) but active regulation is also present via behavioural modulation of activity, habitat use and food intake that affects hydric exposure conditions and thus influences water loss under natural conditions (e.g. Mautz, 1980). This has also been found to likely be reflected in variable daily patterns of water loss (Žagar et al., 2022). Considering that many different aspects of biology of a lizard (behaviour, physiology, morphology, foraging) are involved in the regulation of water balance, it is not surprising that a clear signal of adaptation or plasticity across the altitude could not be found in *G. galloti*. As mentioned previously for the thermal testing results, we caution that these measures may be influenced by testing of physiological parameters outside of each population's native altitude.

Previous studies have shown that in a range of preferred body temperatures of ectotherm organisms, potential metabolic activity increases with increasing body temperature (e.g. Bielen

et al., 2016; Simčič et al., 2014), which was also found in our study. The temperature dependence of potential metabolic activity reflects the concentration of enzymatic complexes (Båmstedt, 1980) and their catalytic properties (Packard, 1971), and higher body temperatures lead to a higher metabolic rate (Angilletta et al., 2002; Žagar et al., 2018). In our study, we detected differences in potential metabolic activity among populations across the altitudinal gradient, with the highest values at the population at 600 m a.s.l., followed by a slight but steady decrease until the highest populations. The lowest values of potential metabolic activity measured at the highest altitude may be a physiological response to environment with more constraints such as higher hypoxia, excessive UV exposure, lower productivity (less vegetation means less food), some of which directly contribute to excess production of reactive oxygen species (ROS). However, there is flexibility by production of ROS due to aerobic respiration in response to changing ATP needs and proton motive force (see Koch et al., 2021); thus, we cannot directly link variation in metabolic activity with potential avoidance strategies of excess ROS production at higher altitudes. We expect to find signals of increasing oxidative stress with higher elevation due to higher UV radiation. While, on the one hand, ROS serve important signalling functions and are not inherently harmful (Zuo et al., 2015), excess ROS can cause oxidative stress. This occurs when oxidative load outweighs antioxidative mechanisms, potentially leading to cell damage and death (Sies, 1985) and under damaging effects of excess ROS, antioxidant mechanisms are simultaneously activated to prevent oxidative stress (Monaghan et al., 2009). We detected higher concentration of carbonyls in *G. galloti* populations at the lowest altitudes, which was contrary to our expectation. Other mechanisms may be involved in avoiding UV radiation stress at highest altitude in *G. galloti*, namely lizards are seemingly more melanistic and have high haemoglobin concentration (RM-P, unpublished data). Further studies are needed to address this variability found in metabolic and antioxidative mechanisms related to environmental stressor variability in the island.

As verified in other studies with telomere length in ectothermic vertebrates, age and telomere length were uncorrelated (Gao & Munch, 2015; Lund et al., 2009). Ageing is known to have an effect on telomere length shortening in many vertebrates (Whittemore et al., 2019), but this has been confirmed mostly for endotherms (also see recent studies arguing for negligible senescence in reptiles; da Silva et al., 2022; Reinke et al., 2022). Ectotherm studies have shown that with ageing, the telomere length either decreases (Scott et al., 2006), increases (Axelsson et al., 2020) or shows a variable pattern as in our system (Gao & Munch, 2015; Lund et al., 2009). This heterogeneity is in part associated with the ability of ectotherms to regenerate telomeres via telomerase activity throughout their life (Olsson et al., 2018). By showing an absence of an effect of age as driver of telomere size, we were able to analyse the trends of telomere length across the altitude. We found differences in RTL among populations at different altitudes that were likely associated with larger differences between the highest altitude population and several of the low altitude

populations; we also found a significant inverse correlation of telomere length with carbonyl metabolite accumulation (these proteins are indicators of physiological stress). Carbonyl protein content was also significantly lower in the highest altitude population where relative telomere length was the highest. Very high altitude poses important challenges to survival, given the often higher intensity of UV radiation, hypoxic conditions or lower food availability. These should lead to signs of metabolic stress, but our results actually suggest the opposite. These results are intriguing and suggest that high-altitude populations can cope with harsh UV and hypoxic conditions without reflecting stress by biochemical markers such as carbonyl.

Indeed, recent literature on telomere lengths suggests that life-history changes may counterbalance telomere attrition driven by stress or accumulation of reactive oxygen species at high altitudes such as a shift to a slower pace-of-life strategy (Giraudeau et al., 2019; Hille & Cooper, 2015), reduced predators or intraspecific competition due to lower population densities and longer hibernation periods (Zamora-Camacho et al., 2013). Like our study, Fitzpatrick et al. (2021) found longer telomeres at high altitudes in the lizard *Niveoscincus ocellatus*, although the specific mechanisms mediating this were not determined. Lower carbonyl protein content at the highest altitude population (indicative of reduced stress) may indicate either metabolic adaptation to these environments, but may also result from the life-history strategies mentioned above that prevent oxidative stress at high altitude. Higher carbonyl content detected in lowland populations could be due to higher stress due to dry conditions. However, it should be noticed that water availability does not vary predictably with altitude (in opposition to other environmental factors, such as oxygen pressure). Therefore, further research is needed to better understand these complex relationships. For instance, laboratory experiments with mice indicate that carbonyl protein content is expected to increase with chronic hypoxia (Lewis et al., 2015).

Several studies have focused on the mechanisms that allow organisms to respond to environmental changes by assessing the relative roles of adaptation and plasticity. Our study highlights the usefulness of adopting multidisciplinary approaches that examine a broad range of traits in a common group of individuals, overcoming simple explanations for adaptation versus plasticity and demonstrating that the way organisms cope with complex environmental stressors may stem from responses that are both complex and trait-dependent. In the particular case of *G. galloti*, a lizard that is very common within its geographically limited insular range, our results suggest that a combination of plasticity and local adaptation acts on several functional traits and contributes to how it interacts with a very heterogeneous environment. In this way, these lizards not only illuminate the evolutionary process underlying functional diversity, but such results also have a value in understanding the complex relationships between organism function and ecosystem processes that are key for planning appropriate conservation strategies (e.g. dispersal corridors, habitat management, translocations) to preserve natural biodiversity of the insular ecosystems. Insular ecosystems

as these, housing endemic species are particularly threatened in the light of climate change while realizing the potential of exposure and resilience of species to these changes will help us to plan their conservation in the future.

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## CONFLICT OF INTEREST STATEMENT

None of the authors have a conflict of interest to disclose.

## DATA AVAILABILITY STATEMENT

Data from this study have been deposited on Zenodo (<https://doi.org/10.5281/zenodo.8132854>).

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## BIOSKETCH

Nina Serén is interested in the adaptive mechanisms that species develop to avoid extinction in our rapidly changing world. The present work constitutes one chapter of her PhD work. Within this framework, she is also interested in the study of population differentiation and adaptation using genomic tools.

**Author contributions:** Nina Serén, Catarina Pinho, Anamarija Žagar and Miguel A. Carretero conceived the study; Nina Serén, Rodrigo Megía-Palma, Miha Krofel, Anamarija Žagar and Miguel A. Carretero conducted the fieldwork and collected data; Nina Serén, Rodrigo Megía-Palma, Tatjana Simčič, Fabio Maria Guarino, Anamarija Žagar and Miguel A. Carretero conducted experiments; Nina Serén analysed data; and Nina Serén led the writing with contributions from all authors.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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