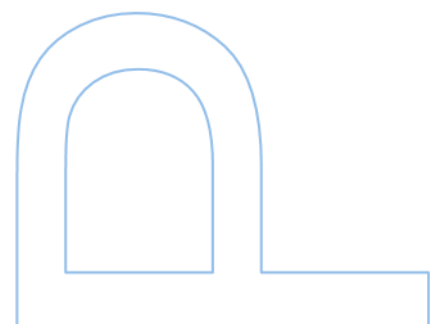
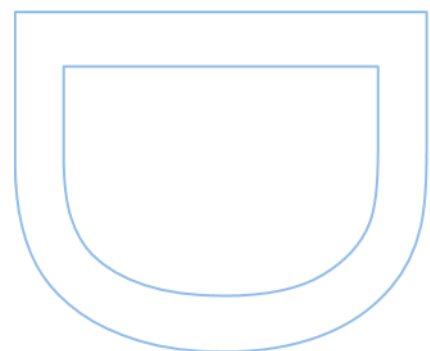
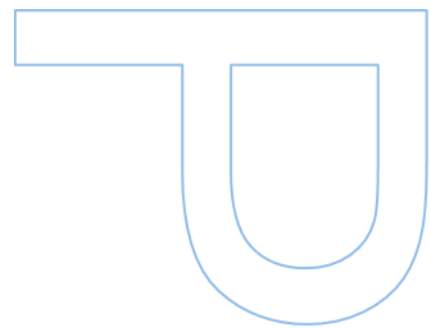




How (will) ectotherms cope with changing environments? A test with a unique model organism under contrasting ecological pressures

Nina Guerra Serén

Doctoral Program in Biodiversity, Genetics and Evolution
Faculdade de Ciências da Universidade do Porto
2022

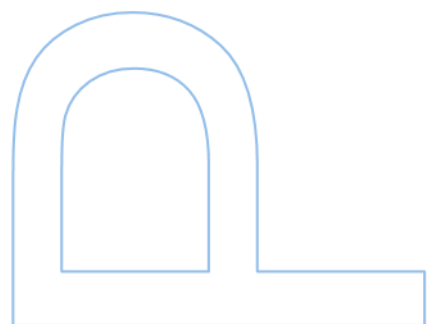
Supervisor

Miguel A. Carretero, Auxiliary Researcher, BIOPOLIS/CIBIO/InBIO, Laboratório Associado, Auxiliary Professor, Faculdade de Ciências, Universidade do Porto

Co-supervisors

Catarina Pinho, Auxiliary Researcher, CIBIO/InBIO, Laboratório Associado, Universidade do Porto

Anamarija Žagar, Scientific Associate, National Institute of Biology, Ljubljana



Declaração de Honra

Eu, Nina Guerra Serén, natural de Porto, residente em Fão com o telefone 913791114, nacional de Portugal, portadora do Cartão de Cidadão nº 13458729 4ZW7, inscrita no Programa Doutoral em Biodiversidade, Genética e Evolução da Faculdade de Ciências da Universidade do Porto declaro, nos termos do disposto na alínea a) do artigo 14.º do Código Ético de Conduta Académica da U.Porto, que o conteúdo da presente tese reflete as perspetivas, o trabalho de investigação e as minhas interpretações no momento da sua entrega.

Ao entregar esta tese, declaro, ainda, que a mesma é resultado do meu próprio trabalho de investigação e contém contributos que não foram utilizados previamente noutros trabalhos apresentados a esta ou outra instituição.

Mais declaro que todas as referências a outros autores respeitam escrupulosamente as regras da atribuição, encontrando-se devidamente citadas no corpo do texto e identificadas na secção de referências bibliográficas. Não são divulgados na presente tese quaisquer conteúdos cuja reprodução esteja vedada por direitos de autor.

Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

Nina Guerra Serén

Vairão, dia 22 de Julho de 2022



Acknowledgements

Working on a PhD thesis is more than building up a complete and understandable (at the very least, in the field of expertise) piece of storytelling science. Many PhD students probably only faintly remember the initial challenges and drawbacks (that looked absolutely immense at the time) of their PhD workplan during the work of their first couple of years. The reason for this may be due to the final effort of finishing the thesis that, dare I say, in many cases is something completely out of any personal expectations, even if you are warned against it before it all starts. In my case, important global events coincided to happen right in the middle and in the end, which made my path to finish the PhD a bit harder than expected, either by directly affecting my work or by making me shift my attention to worldwide or European calamities. 1) The pandemic of Covid-19 in the last couple of years, 2) the greatest war in Europe since World War II started in February 2022, almost exactly 5 months before my thesis delivery deadline and finally 3) climate change, which overrides these last two global catastrophes in the news due to subsequent and rather intense heatwaves in June and July of 2022. The greatest challenge of it all was that scientific work really had to change its pace back and forth way many more times than recommended.

Regardless of how humans have been taking care of themselves and this planet, and the horrendous consequences of epidemics, war and climate change, the fact is that our life values and work goals remain the same. As such, I still delivered this thesis and I was still able to maintain all that is most valuable in life to me – family, friends and acquaintances, luckily in a free (and war-free) country, having a garden, health and lastly, some accomplished work. And all of this would not be possible without all people and plants that far or close, made part of my life and thus helped me in some way to have a great life these last years. For them I will give my few but GREAT thanks to, and starting by work-related people.

1- Friends

I will start with all my supervisors, because they were really great Friends. They understood whatever delays and struggles of not being able to work outside normal work hours, or overwork due to family at home. Miguel Carretero, my main supervisor, is the best storyteller I know in science, and I am pretty convinced he will forever remain in that

status. I had him as a co-supervisor during my Master thesis although in a different topic, so I knew him well and really wanted to continue my scientific path with him. He was always very attentive, specially given his own amount of work, and had all the time a very positive comment and would help me gain moral when I needed. Also I will never forget how he opened a slot for field work in Tenerife collecting samples from what was back than only a PhD project without project grant to back it.

A big friend also is Catarina Pinho, my co-supervisor and important genetics advisor for fruitful discussions and creative input into ideas for the work, never ceasing to give a lot of moral and support about my doubts along the PhD. She was a great help also when I needed some confidence using R in some of the analyses, and I hope I can retribute some of this help with my recently gain knowledge in genomics whenever she needs.

To Anamarija Žagar, my other co-supervisor: without her pushing me into things, and without her feedback and plans on how to get things done, things would have been more complicated. Her insight on certain lab techniques was also of great importance to enrich my thesis, and my own lab work experience, since laboratory work is very dear to me. I started to know her best in the very first field trip travel to Tenerife, where we were both in the lab working when I received the news of the approval of the doctoral grant, and shared that incredible moment together (as I was also her first official PhD student).

Now I will list what could easily be a fourth supervisor, Fabio Maria Guarino from University of Naples Federico II, one of the greatest skeletochronology specialists in amphibians and reptiles: he taught me with immense patience and detail his knowledge on this “art”. My time in Naples with him seems very far away, but since then he had a great amount of work revising my results and commenting with incredible detail and patience an immense list of samples for age estimation.

One person that without his help and hard work I would not have so many valuable fresh samples to work with right from the start of my work, is the real expert on my lizard study species, Dr Rodrigo Mégia-Palma. He is a walking encyclopaedia of these and other lizards, although officially he is a well-known expert in lizard parasites. I will never know so much as he does about good practices in field work, and will never beat him at Carcassone (the board game) either.

Thanks also to Miguel Nogales in CSIC who welcomed us in Tenerife for our first field work carried out in the island. CTM (Centro de Testagem Molecular) technicians in

CIBIO were also very helpful to provide me with guidance and sometimes performing some of the laboratory work, with special thanks to Maria Magalhães, Sandra Afonso and Susana Lopes, the last one for giving me a valuable slot in the lab under very restraining access conditions due to the pandemic, back when I was pregnant and needed to rush work before maternity leave.

Thank you also to my long-term friends from my bachelor at Porto's Faculty of Sciences, Joana Bessa, recent PhD holder from Oxford, and Raquel Costa, recent PhD holder from Osaka University, both with expertise in behaviour of great apes, for hearing me complaining about my work troubles that had nothing to do with apes, but that of course they complied to listen and tried to help anyway.

2 – Institutions

I would like to thank FCT – Fundação para Ciência e Tecnologia for the financial support through the PhD grant SFRH/BD/128612/2017 and 3 month-extension grant (COVID/BD/152580/2022). I would like also to acknowledge my host institution as well as others that provided the conditions and space to conduct my work there during short visits CIBIO-InBIO Research Centre in Biodiversity and Genetic Resources, FCUP – Faculdade de Ciências da Universidade do Porto; IPNA-CSIC (Spanish National Research Council - Department of Agrobiology and the Environment at La LAGUNA in Tenerife, Spain; the National Institute of Biology at Ljubljana, Slovenia NIB; and University of Naples Federico II.

3 – Family

A deep and special thanks to my close family, parents, grandmother and teen brother that do not know almost anything about my thesis but still should be listed. My kids, Gil and Rafael that know even less about the thesis but that, like every son, can completely worn us much more times that we would like, and also boost us with happiness in a way nothing else in the world can. Last, but not least, to my partner in life and the dad of my two kids, Pedro Andrade which is part of me and part of everything. He also, regrettably in my way of view, got to know too much about my work but at least I know he enjoyed part of it. Thank you Pedro for being always present and always so important to my life.

Abstract

How do organisms cope with environmental change? This is a question of critical importance, not only as a topic of broad interest in evolutionary biology, but also due to an increase in research and public focus in matters related to ongoing anthropogenic climate change. When faced with shifting environments, organisms can respond either by individually modifying the expression of a given trait (phenotypic plasticity) or by changes in the frequency of alleles over generations (genetic adaptation). To thoroughly understand these processes, studies focusing on multiple aspects of the biology of species inhabiting strong environmental gradients are needed. This thesis is focused on studying the physiological, metabolic and genomic mechanisms of response to environmental change used by a unique lizard species, *Gallotia galloti*. This species occurs throughout the island of Tenerife (Canary Islands), where it is found in a variety of habitats. In particular, it colonized a steep altitudinal gradient along the Teide volcano, from sea level up to an altitude of over 3,700 metres. Apart from a general introduction and final discussion, this thesis includes three main chapters in which novel research work on this topic is presented.

The first of these is focused on describing the responses caused by changing stressful abiotic conditions, i.e. temperature, partial oxygen pressure, UV radiation associated with altitude in *G. gallotia*. To do this, we sampled lizards in several localities along the altitudinal gradient of the Teide and collected morphological, physiological and biochemical data. The main differences that were found were related to lower biochemical signals of stress in highland lizards (longer relative telomere length, decreased carbonyl content). Thermal aspects of the biology and age structure did not vary with altitude, while there were some differences along the gradient in hydric biology but non-depended from altitude and most likely linked with local variation in body size and precipitation regimes. Overall results suggest that, contrary to expectations, lizards at high altitudes seem comparatively under less stress; also, despite their very contrasting environments, lizards from different altitudes maintain the same thermal optima, which points to a scenario of conservativeness of these parameters by lizards. Plasticity should thus have a role in their colonization of harsh high altitudinal environments.

The second research work investigated patterns of genetic variation in populations of *G. gallotia* living at the extremes of the altitudinal gradient. To do this a

reference genome was first assembled using linked-read technology, with which one of the most complete and contiguous lizard genomes was produced to date. Genomic comparisons using whole-genome re-sequencing data suggest relatively reduced overall differences, but highlight the role of selection on genes linked to response to hypoxia as an important driver of genetic adaptation. In contrast to other abiotic factors that vary with altitude (such as temperature, or UV exposure), hypoxia is less likely to be tackled through behavioral changes or phenotypic plasticity alone.

For the final research work, the focus was shifted to the evolutionary axis by comparing two deep lineages of *G. galloti*: *G. g. galloti* (living on the Southern side of Tenerife) and *G. g. eisentrauti* (found on the Northern Side). Microclimatic differences between the two sides of the island (more humid in the North, arid in the South) were predicted to drive differences in thermal and hydric physiology and metabolism in the two sister lineages. Testing revealed that *G. g. eisentrauti* selected for lower temperatures and had higher rates of water loss; metabolism was however similar. Little variation was found between populations at different altitudes within each lineage indicating that adaptation is past rather than recent. These results confirm the initial expectations, but also hint at why only *G. g. galloti* managed to colonize the high summit of the island.

Overall, the research performed for this thesis suggest that, in an insular lizard that inhabits a heterogeneous environment with different microclimates and a steep altitudinal gradient, a combination of phenotypic plasticity and genetic adaptation had likely contributed for the colonization success of this species that is widespread in Tenerife. Ectothermic vertebrates have been frequent models in studies focused on their responses to environmental changes, in particular those focused on altitude as a proxy of environmental variation. However, the work performed for this thesis provides one of the more comprehensive approaches to this question, based on a large variety of techniques to describe phenotypic and genomic variation underlying responses to environmental change.

Keywords: Adaptation, Telomere, Plasticity, Ecophysiology, Physiology, Genome, Lizard, Tenerife island, Environmental change

Resumo

Como é que os organismos lidam com alterações ambientais? Esta é uma questão de extrema importância, não apenas como tópico de amplo interesse em biologia evolutiva, mas também devido à crescente atenção dada por investigadores e o público em geral a matérias relacionadas com alterações climáticas de origem antropogénica. Perante mudanças ambientais, os organismos podem responder ora por mudanças individuais na expressão de uma característica (plasticidade fenotípica) ora por mudanças nas frequências de alelos ao longo de gerações (adaptação genética). Para compreender estes processos são necessários estudos focados em múltiplos aspetos da biologia de espécies que habitam gradientes ambientais. Esta tese tem como foco o estudo dos mecanismos fisiológicos, metabólicos e genómicos de resposta às alterações ambientais utilizados por uma espécie de lagarto, *Gallotia galloti*. Esta espécie ocorre ao longo da ilha do Tenerife (Ilhas Canárias), onde é encontrada em vários habitats. Em particular, colonizou um gradiente altitudinal bastante acentuado ao longo do vulcão do Teide, desde o nível do mar até uma altitude próxima dos 3.700 metros. Para além de uma introdução geral e discussão final, esta tese inclui três capítulos nos quais se apresentam trabalhos de investigação inéditos.

O primeiro destes três trabalhos está focado na descrição de respostas causadas pela flutuação do stress a condições abióticas, i.e. temperatura, pressão parcial de oxigénio e radiação UV, associadas à altitude em *G. galloti*. Para isto, amostrámos lagartos em várias localidades ao longo do gradiente altitudinal do Teide e recolhemos dados morfológicos, fisiológicos e bioquímicos. As principais diferenças encontradas estavam relacionadas com baixos sinais de stress bioquímico em lagartos de grande altitude (maiores comprimentos relativos de telómeros, menores níveis de carbonilo). Aspetos térmicos da biologia e estrutura etária não variaram com a altitude, enquanto que se evidenciaram algumas diferenças ao longo do gradiente em biologia hídrica, mas estas não dependiam da altitude, sendo mais provável estarem ligadas à variação local de tamanho corporal e regimes de precipitação. No geral estes resultados sugerem que, ao contrário da expectativa, lagartos de grande altitude parecem estar comparativamente sob menos stress; e também, apesar dos ambientes bastante contrastantes, lagartos de altitudes diferentes mantêm os mesmos níveis ótimos do ponto de vista térmico o que aponta para um cenário de regulação ativa destes parâmetros pelos lagartos. A plasticidade deve, portanto, ter um papel importante na colonização de ambientes de alta altitude por estes lagartos.

O trabalho seguinte de investigação averiguou padrões de variação genética em populações de *G. galloti* oriundas dos extremos do gradiente altitudinal. Para fazer isto, foi produzido um genoma de referência com recurso a tecnologia *linked-read*, obtendo-se um dos mais completos e contíguos genomas de lagarto existentes. Comparações genómicas com recurso a dados de re-sequenciação de genomas completos sugerem um cenário geral de pouca diferenciação entre populações, mas destacam o papel da selecção em genes ligados a respostas à hipóxia como um importante motor da adaptação genética. Contrastando com outros fatores abióticos que variam com altitude (tal como temperatura, exposição a radiação UV), o stress por hipóxia é menos provável de se conseguir evitar através de alterações comportamentais ou plasticidade fenotípica

Para finalizar os trabalhos de investigação, o foco centrou-se na comparação entre duas subespécies de *G. galloti*: *G. g. galloti* (natural da parte sul do Tenerife) e *G. g. eisentrauti* (encontrada no lado norte). As diferenças microclimáticas entre as duas vertentes da ilha (mais húmida a norte, árida no sul) suscitaram a hipótese de que surgissem diferenças na fisiologia térmica e hídrica, assim como no metabolismo, nas duas linhagens. Testes revelaram que *G. g. eisentrauti* selecciona temperaturas mais baixas e perde mais água; o metabolismo, no entanto, revelou-se semelhante. Pouca variação foi encontrada entre populações a diferentes altitudes dentro de cada linhagem, o que indica que a adaptação é mais antiga. Estes resultados confirmam não só a expectativa inicial mas também parecem explicar o porquê de apenas *G. g. galloti* ser capaz de colonizar os habitats de maior altitude da ilha.

Em geral, a investigação levada a cabo sugere que, num lagarto insular que habita ambientes heterogéneos na presença de microclimas diferentes e um gradiente altitudinal abrupto, a combinação de plasticidade fenotípica e adaptação genética provavelmente contribuiu para o sucesso da colonização desta espécie que hoje se encontra por toda a ilha de Tenerife. Os vertebrados ectotérmicos são modelos frequentes em estudos focados nas respostas biológicas a alterações climáticas, com particular destaque a estudos em que a variação altitudinal é usada como proxy de variações ambientais. No entanto, o trabalho produzido para esta tese proporciona uma das mais abrangentes abordagens a esta questão, baseada numa grande variedade de técnicas para descrever a variação fenotípica e genómica subjacente às alterações ambientais.

Palavras-chave: Adaptação, Telómeros, Plasticidade, Ecofisiologia, Fisiologia, Genoma, Lagarto, Ilha de Tenerife, Mudanças ambientais

Table of contents

Acknowledgements	v
Abstract	ix
Resumo	xi
Table of contents	xiii
List of figures	xv
List of tables	xxi
List of abbreviations	xxv
Research articles not included in thesis.....	xxvii
Chapter I – General introduction	1
1.1. How to avoid extinction? The roles of phenotypic plasticity and genetic variation in adaptation to global environmental change	2
1.2. Altitudinal gradients as a means to explore organismal responses to environmental change	7
1.3. Adaptations to altitude in ectotherms, with a focus on lizards	11
1.4. <i>Gallotia galloti</i> , an endemic insular ectotherm that inhabits a steep altitudinal gradient in Tenerife (Canary Islands)	14
1.5. Objectives and organization of the thesis	20
1.6. References.....	22
Chapter II – Plastic functional responses in a lizard living along a steep altitudinal gradient	36
2.1. Abstract	38
2.2. Introduction	39
2.3. Methods.....	43
2.4. Results.....	51
2.5. Discussion.....	58
2.6. Acknowledgements	62
2.7. References.....	63
2.8. Supplementary materials.....	75
Chapter III – Selection on hypoxia-associated genes mediates altitudinal adaptation in an insular lizard.....	89
3.1. Abstract	91
3.2. Introduction	92

3.3. Methods.....	96
3.4. Results.....	101
3.5. Discussion.....	109
3.6. Acknowledgements	112
3.7. Data availability	113
3.8. References.....	114
3.9. Supplementary materials.....	125
Chapter IV – Ecophysiology and metabolic potential of two lizard lineages inhabiting contrasting habitats within an island.....	129
4.1. Abstract	131
4.2. Introduction	132
4.3. Methods.....	135
4.4. Results.....	140
4.5. Discussion.....	144
4.6. Acknowledgements	147
4.7. References.....	148
4.8. Supplementary materials.....	153
Chapter V – General discussion	158
5.1. Phenotypic plasticity helps to cope with drastic environmental changes.....	159
5.2. Selection on hypoxia-associated genes may explain adaptation to altitude	161
5.3. Knowing the evolutionary history of <i>G. galloti</i> is necessary to test functional hypotheses	164
5.4. Concluding remarks	166
5.5. References.....	168

List of figures

Cover image credits: Siegfried Troidl (CC BY-NC 4.0)

Figure 1.1. When phenotypic change is necessary to respond to changes in environment, organisms can either modify a trait through phenotypic plasticity (trait expression is not determined by an individual's genotype, typically a short-term response) or adaptation (when shifts in frequencies of a causal allele drive changes in the trait, typically required for long-term response). Image adapted from Behm & Kiers (2014).

Figure 1.2. Increasing altitudes are associated with decreasing atmospheric oxygen pressure. Multiple studies have shown this to be one of the most important abiotic constraints for organisms living in high altitude habitats. Image by Brendan Scott (used under a CC-BY-ND license).

Figure 1.3. Map of the Canary Islands archipelago and the seven extant *Gallotia* species. Photo credits: *G. atlantica* (Sébastien Sant, CC-BY-NC 4.0); *G. stehlini* (Siegfried Troidl, CC-BY-NC 4.0); *G. intermedia* (Siegfried Troidl, CC-BY-NC 4.0); *G. galloti* (Siegfried Troidl, CC-BY-NC 4.0); *G. bravoana* (Cristoph Moning, CC-BY 4.0); *G. caesaris* (Haplochromis); *G. simonyi* (Domingo Trujillo González).

Figure 1.4. The two main lineages of *Gallotia galloti* that can be found in Tenerife: *G. g. eisentrauti* and *G. g. galloti*. Dashed line on the map of Tenerife signals the approximate delimitation of their distribution within the island. Below, landscape of Tenerife in the southern part of the island (drier) showing the Teide volcano in the background. Credits for photos: *G. g. eisentrauti* (Jan Ebr, CC-BY 4.0); *G. g. galloti* (Siegfried Troidl, CC-BY-NC 4.0); Tenerife (Ingo Mehling).

Figure 2.1. 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. Photo by Nina Serén. (b) Geographical location of the island of Tenerife in the Canary Islands archipelago and a close-up of island topography showing the sampling sites. (c) Location of sampling sites along a

topographic profile of the island of Tenerife from the sea to the highest point of the island (following the southeast sampling direction of the sampling).

Figure 2.2. (a) Example of age estimation based on skeletochronology of growth rings in the phalanx of one analysed sample of *G. galloti*. Abbreviations: HL: hatching line; RL: reversal line; f: false LAG (line of arrested growth); 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) 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).

Figure 2.3. 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 thermoregulate; 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 weight due to water loss. (c) Location profiles of thermal preferences along the different periods of the day. (d) Location profiles of instantaneous water loss (EWL_i) along the different periods of the day. (e) Location profiles of accumulated water loss (EWL_a) along the different periods of the day. In each graph, populations are arranged (left-to-right) from the lowest altitude to the highest altitude. All variables are controlled for size and log-transformed.

Figure 2.4. 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 distinct 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.

Figure S2.1. – Determination of real-time qPCR efficiencies (mean of all 96-well plates) of the reference gene (18S) and the telomere amplified fragments (telom). The

corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{-1/\text{slope}}$ (Rasmussen, 2001).

Figure S2.2. Boxplot showing variation of size in the form of SVL (snout-vent length), under a log 10 transformation along locations which are ordered by increasing altitudes from 100m to 3550m.

Figure S2.3. Boxplot showing variation of weight using a log10 transformation along locations which are ordered by increasing altitudes from 100m to 3550m.

Figure 3.1. Altitudinal sampling design to study adaptation in an insular lizard. (a) The study species, *Gallotia galloti*, adult male. Photo by Jörg Hempel (used under a CC BY-SA 3.0 DE license). (b) Geographical location of the Canary Islands archipelago and the island of Tenerife (with labelled sampling sites for whole-genome sequencing). The solid black line drawn on the south side of the island indicates the topographic profile in the following panel. The broader map of Europe and North Africa was taken from Natural Earth (<https://www.natureearthdata.com>). (c) Location of sampling sites along an example topographic profile of the south side of the island of Tenerife.

Figure 3.2. Population genomics of high and low-altitude *Gallotia galloti*. (a) Pairwise values of the fixation index (F_{ST}) between pools of samples from the four populations in the study. (b) Box-plots with distribution of genome-wide values of nucleotide diversity (π , %). (c) Box-plots distribution of genome-wide values of Tajima's D , with middle line representing the mean, box the 1st and 2nd quantiles and lines the 3rd and 4th quantiles. The three statistics were calculated for non-overlapping windows of 100 kb.

Figure 3.3. Genome-wide signatures of selection potentially associated with high-altitude adaptation in *Gallotia galloti*. (a) Fixation index (F_{ST}) between a high-altitude population and the combined data from three low-altitude populations. (b) Ratio between the nucleotide diversity (π , %) of low-altitude and high-altitude populations. (c) Differences in the allele frequency spectrum of low and high-altitude populations as calculated with Tajima's D . (d) De-correlated composite of multiple statistics (DCMS),

calculated with values from the three statistics in panels a-c. The dashed line corresponds to the threshold indicating the top 0.1% windows in the distribution (windows above it were considered as representing strong evidence of selection). Each dot on the Manhattan plots represents one 50 kb window (windows were calculated with overlap of 12.5 kb). Colours represent alternating genomic scaffolds.

Figure 3.4. Localized genomic signatures of adaptation to altitude in *Gallotia galloti*. Detailed view of patterns of genetic differentiation (F_{ST}), variation (π , %) and allele frequency spectrum (Tajima's D) for the two most important outlier regions in our genome scans. Each dot represents a non-overlapping 2.5 kb window (results calculated using the same parameters as for the genomic scan). Annotated genes in these regions are shown on top (gene names adapted from the human orthologues).

Figure S3.1. Comparison between the *Gallotia galloti de novo* assembly and other published lacertid genomes (*Lacerta agilis*, *Lacerta bilineata*, *Lacerta viridis*, *Podarcis muralis* and *Zootoca vivipara*). (a) Size (Mb) of the top 55 genomic scaffolds of *G. galloti* (all scaffolds larger than 100 kb). (b) Scaffold N50. (c) Contig N50. (d) BUSCO scores (aligned to the tetrapoda_odb10 database).

Figure S3.2. Genetic differentiation (fixation index, F_{ST}) between high-altitude (CT) and low-altitude (GR, P, SA) populations of *G. galloti*. Each dot corresponds to a 50 kb window, with successive windows having a 12.5 kb overlap. Alternating colors indicate the different genomic scaffolds.

Figure 4.1. Altitudinal sampling design to study ecophysiological and metabolic traits in *Gallotia galloti galloti* and *G. g. eisentrauti*. (a) The geographic context of the Canary Islands archipelago, with an arrow indicating the island of Tenerife. (b) Map of Tenerife showing sampling sites; (c) Map of Tenerife with schematic representation of a ring-shaped area (oblique hatching) that illustrates the approximate position of a frequent ground-level cloud area, which has low population densities of *G. galloti*; image reproduced from Thorpe & Richard (1989) (d) Map of Tenerife retrieved from Albaladejo-Robles et al., 2022 showing the main bioclimatic belts and main patches of forest cover; image based on Rivas-Martínez (1987)

Figure 4.2. Box-and-jitter plots showing patterns of: (a) thermoregulatory preferences (T_p) along different periods of the day by lineage and altitude; (b) accumulated water loss (EWL_a) along the different periods of the day by lineage and elevation; (c) instantaneous water loss (EWL_i) along the different periods of the day; (d) potential metabolic activity (PMA) along three distinct testing temperatures (29 °C, 33 °C and 37 °C) by lineage and altitude.

Figure S4.1. Box-and-jitter plot of log-transformed snout-vent length (SVL, a proxy of body size) for *Gallotia galloti eisentrauti* and *G. g. galloti*, collected at three different locations each (three altitudinal points).

Figure S4.2. Box-and-jitter plot of log-transformed weight for *Gallotia galloti eisentrauti* and *G. g. galloti*, collected at three different locations each (three altitudinal points).

Figure S4.3. Plots with raw data for preferred temperatures (T_p , top) and accumulated water loss (EWL_a , bottom) for *Gallotia galloti eisentrauti* and *G. g. galloti*, collected at three different locations each (three altitudinal points).

List of tables

Table 2.1. Number of individuals from each sampling location in relation to the analysed variables. Abbreviations: T_p - preferred temperature; EWL - evaporative water loss; PMA - potential metabolic activity; RTL - relative telomere length.

Table 2.2. Results of different analyses of variance (ANOVA) to assess variation in ecophysiological, metabolic and life-history variables in relation to sampling location in *G. galloti*. Abbreviations: T_p - preferred temperature; EWL_i - instantaneous evaporative water loss; EWL_a - accumulated evaporative water loss; PMA - potential metabolic activity; RTL - relative telomere length; SVL - snout-vent length; LOC - sampling location. Showing Generalized eta squares for Repeated Measures ANOVA and Sum squares for ANCOVA and 2-way ANOVA.

Table S2.1. Standard statistics for all non-transformed studied variables. Abbreviations: SVL – Snout-vent length; T_p - preferred temperature; EWL_i - instantaneous evaporative water loss; EWL_a - accumulated evaporative water loss; PMA - potential metabolic activity; RTL - relative telomere length. * All repeated measures; ** only last EWL_a measure.

Table S2.2. Telomere efficiency qPCR amplification calibrator sample reaction tests. C_q is the quantification cycle value.

Table S2.3. Same as Table 2.1 from Chapter II, but sample numbers have already been filtered for statistical analysis (after outlier removal or non-validated age estimation score for this variable). Abbreviations: SVL - Snout-vent length; T_p - preferred temperature; EWL_i - instantaneous evaporative water loss; EWL_a - accumulated evaporative water loss; PMA - potential metabolic activity; RTL - relative telomere length.

Table S2.4. SVL pairwise differences using Benjamini-Hockberg *P*-value correction; in bold are significant *P* values

Table S2.5. Weight pairwise differences using Benjamini-Hockberg *P*-value correction; in bold are significant *P* values

Table S2.6. Ordinary Least Squares Regression of age and log-transformed size (snout-vent length; SVL).

Table S2.7. Pairwise differences tests on EWL_a using Benjamini-Hockberg *P*-value correction.

Table S2.8. Pairwise differences tests on EWL_i using Benjamini-Hockberg *P*-value correction.

Table S2.9. Pairwise differences tests on PMA using Benjamini-Hockberg *P*-value correction.

Table S2.10. Catalase antioxidant activity pairwise differences with non-pooled SD using Benjamini-Hockberg *P*-value correction.

Table S2.11. Carbonyl metabolites pairwise differences with non-pooled SD using Benjamini-Hockberg *P*-value correction; in bold are significant *P* values

Table S2.12. RTL pairwise differences with non-pooled SD; using Benjamini-Hockberg *P*-value correction; in bold are significant *P* values

Table 3.1. Genomic intervals with evidence of selection to high-altitude in *Gallotia galloti*, highlighted from a composite selection statistic (DCMS) and ordered from highest to lowest DCMS value. Protein coding genes in each interval were retrieved from the *G. galloti* genome annotation, considering a 100 kb buffer either side of the genomic interval with evidence of selection, and were here given gene names from their human orthologues. Protein coding sequences with no known orthologues (marked with *) were given a specific code, following the annotation.

Table S3.1. Summary statistics of the *de novo* reference genome assembly and annotation of *Gallotia galloti*.

Table S3.2. Summary statistics of the whole-genome pool-sequencing dataset.

Table 4.1 – Descriptive statistics of the collected data of populations from different sampling localities. Sample size shown for the analyses of thermogradient preferences (T_p) and accumulated evaporative water loss (EWL_a) include the number of individuals after outlier removal.

Table 4.2. Results of different analyses of variance (ANOVA) to assess variation in ecophysiological and metabolic traits in relation to subspecies and relative elevation between sampled locations. Abbreviations: T_p - preferred temperature; EWL_i - instantaneous evaporative water loss; EWL_a - accumulated evaporative water loss; PMA - potential metabolic activity. Showing Generalized eta squares for Repeated Measures ANOVA and Sum squares for ANCOVA and 2-way ANOVA.

Table S4.1. Pairwise differences between all sampling localities for snout-vent length (SVL, proxy of body size). *P*-values were adjusted using the Benjamini-Hochberg method. Localities for *Gallotia galloti galloti*: EM, GR, VL. Localities for *G. g. eisentrauti*: BV, TA, LL.

Table S4.2. Pairwise differences between all sampling localities for weight. *P*-values were adjusted using the Benjamini-Hochberg method. Localities for *Gallotia galloti galloti*: EM, GR, VL. Localities for *G. g. eisentrauti*: BV, TA, LL.

List of abbreviations

a.s.l. – above sea level

ADRA1A – Adrenoceptor alpha 1A

BMPER – BMP-binding endothelial regulator

BNIP3L – BCL2-interacting protein 3-like

bp – base pairs

CDS – Coding sequence

CI – Confidence interval

DCMS – de-correlated composite of multiple signals

DNA – Deoxyribonucleic acid

DPYSL2 – Dihydropyrimidinase-like 2

EPAS1 – Endothelial PAS domain-containing protein 1

EWL – Evaporative water loss

EWL_a – Accumulated evaporative water loss

EWL_i – Instantaneous evaporative water loss

F_{ST} – Fixation index

Gb – 1,000,000,000 base pairs

kb – 1,000 base pairs

LAG – Line of arrested growth

Mb – 1,000,000 base pairs

mM – Milimolar

Mya – Millions of years ago

N50 – Minimum contig/scaffold length needed to cover 50% of the genome

O₂ – Oxygen

OLS – Ordinary least squares

PCR – Polymerase chain reaction

PMA – Potential metabolic activity

qPCR – Quantitative polymerase chain reaction

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RTL – Relative telomere length

SNP – Single nucleotide polymorphism

SVL – Snout-vent length

T_p – Preferred body temperature

UV – Ultraviolet radiation

W_0 – Initial body weight

ΔAF – Allele frequency differences

π – nucleotide diversity

Research articles not included in thesis

The following research article was published during the course of the doctoral work. Since it constitutes research performed outside the framework of the main topic, it was not included in the thesis.

Megía-Palma, R., Arregui, L., Pozo, I., Žagar, A., Serén, N., Carretero, M. A., & Merino, S. (2020). Geographic patterns of stress in insular lizards reveal anthropogenic and climatic signatures. *Science of the Total Environment*, 749, 141655.

Chapter I – General introduction

1.1. How to avoid extinction? The roles of phenotypic plasticity and genetic variation in adaptation to global environmental change

Traditional definitions of biodiversity highlight its multilevel aspect, since it comprises not only the number of different species, but also their interaction with the ecosystems and genetic variation within and between species (Noss, 1990; Maclaurin & Sterelny, 2008). Biodiversity is widely recognized as a key component for the capacity for ecosystems to provide human society with supporting, provisioning, regulating and cultural services, which makes the preservation of biodiversity of extreme importance (Millenium Ecosystem Assessment (2005); Duraiappah et al., 2005). However, natural populations are increasingly threatened, as the conservation status of species across many major taxonomic groups deteriorates (Ceballos et al., 2020; IUCN, 2021), and even species that are typically considered to be abundant and of low concern have experienced important declines in population size and distribution range (Ceballos et al., 2017). Several causes have been linked to this drop in biodiversity levels, the majority of which are associated with increasing anthropogenic activity, such as habitat loss, climate change, introduction of invasive species, overexploitation or pollution (Sodhi et al., 2009). This ongoing “extinction crisis” is so severe that many studies have started to refer to it as the “sixth mass extinction” (Wake & Vredenburg, 2008; Barnosky et al., 2011; Schachat et al., 2021; Cowie et al., 2022), since extinction rates are similar to those estimated for five previous major extinction events in the Phanerozoic Eon (at the end of the Ordovician, Devonian, Permian, Triassic and Cretaceous periods). Rates of extinction are not uniform between biogeographical regions, habitats and taxonomic groups (Pimm et al., 2014; Payne et al., 2016; Le Roux et al., 2019) which provides an opportunity to understand why some species are more threatened, and what response strategies can be used by species to cope with environmental change.

Understanding the means by which life forms diversify to cope with environmental change is nowadays a topic of great research interest. This is important for gaining basic knowledge on a species’ functional biology (which explores the factors that shape the occurrence of a given trait on an organism), which can then inform on how species will respond under different scenarios of change. With this information, it could be possible to devise effective conservation strategies that take into account species’ life history (Supple & Shapiro, 2018). Natural populations of different species respond very differently to environmental change, as species may: 1) adapt to the new dominant

conditions; 2) persist in suboptimal conditions potentially losing range and population; 3) shift in range, or 4) go extinct and all of these responses will depend on other species effects such as competitors, prey, predators and parasites (Davis et al., 2005). The first straightforward response of populations may be distributional shifts, by which populations track locations with habitat suitability without changing their fundamental niche (e.g., Davis & Shaw, 2001; Midgley et al., 2003; Perry et al., 2005). However, these responses may not always be applicable because distributional range shift opportunities may be spatially constrained (for example insular endemic species are highly geographically constrained). Furthermore, such distributional responses depend on the dispersal ability of organisms. Highly mobile organisms such as migratory birds, insects or large mammals can easily shift ranges, even within short time periods (see Areta et al., 2021 for an extreme example). Sometimes rates of dispersal may even vary in taxa with clear ecological links. For example, dispersal ability may be extremely restricted in small vertebrates such as lizards, but not necessarily in their parasites, especially if they are transmitted by vectors with high-mobility and influence of climate change (for example, mosquitos, Elbers et al., 2015).

When range shifts alone are not a viable alternative to evade the effects of environmental change, phenotypic change also becomes necessary (although both processes likely have complementary roles during response to change, Catullo et al., 2019). Long-term changes in phenotypes (i.e. evolutionary change, Figure 1.1) are likely mediated by genetic changes, specifically changes in the frequencies of alleles at loci of functional importance (protein-coding or regulatory sequences). Several examples of environmental change driving adaptive evolution have been described in detail, including the identification of specific loci and the alleles that underlie adaptive traits. One of them is one of the better known textbook examples of natural selection, the British peppered moths (*Biston betularia*). In this system, increased pollution associated with the Industrial Revolution augmented the selective advantage of melanic forms, which gained enhanced camouflage against darkened tree trunks. The mutation causing this effect was described by van't Hof et al. (2016) as a transposable element insertion within an intronic region of gene *cortex* in melanic moths that causes an increased expression of this gene, and thus the differentiated phenotype. Another example of adaptive evolution linked to human-driven environmental change is the case of winter coat colour in hares (*Lepus* spp.). Many species of this genus undergo seasonal coat colour changes to match the environment (brown in summer, white in winter). Territories that are evidencing reduced snow cover due to climate warming promote selection for higher frequency of hares that do not moult to white coat colour during winter (Jones et al., 2020). Variation

in winter coat colour has been demonstrated by Jones et al., (2018) to be caused by regulatory variation controlling seasonal expression of the *ASIP* gene (agouti-signalling protein). In these examples, the interaction between organism and environment is also affected by the interactions with other members of the biotic community (in both cases predators), which highlights that detailed descriptions of the factors promoting adaptation should always take care to consider multiple elements of an organism's ecological interactions (biotic and abiotic). Ultimately, only natural selection can drive long-term adaptation to a constantly changing environment, but importantly it depends on the availability of beneficial alleles in a population, which can only exist either via new genetic mutations or introduced by gene flow from other populations. Although evolution by standing genetic variation is also commonly observed, and promote rapid shifts in phenotype (Barrett & Schluter, 2008), this may be more likely if an unstable environment promotes the coexistence of different genetic variants. In many circumstances of sudden change, beneficial alleles may not be readily available.

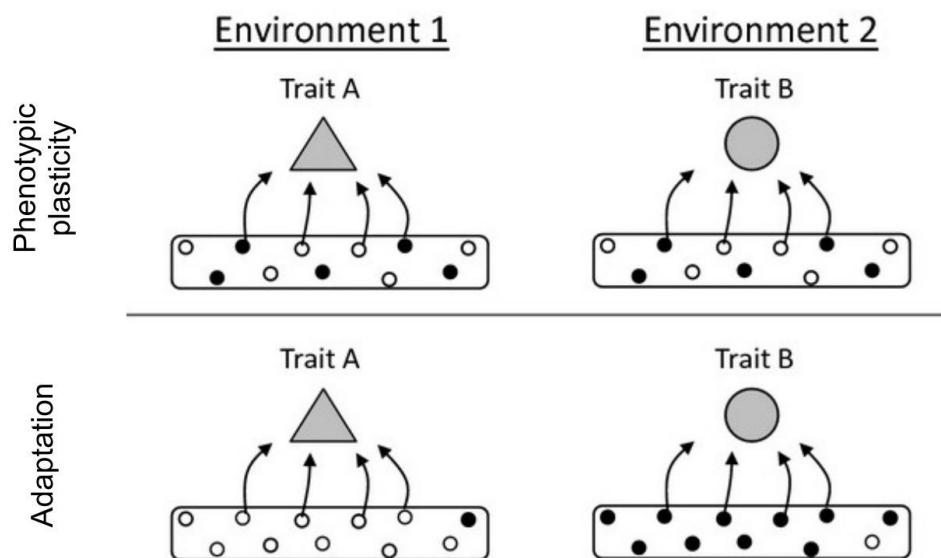


Figure 1.1. When phenotypic change is necessary to respond to changes in environment, organisms can either modify a trait through phenotypic plasticity (trait expression is not determined by an individual's genotype, typically a short-term response) or adaptation (when shifts in frequencies of a causal allele drive changes in the trait, typically required for long-term response). Image adapted from Behm & Kiers (2014).

However, the strength of the link between phenotype and genotype will also determine how effective natural selection will be in driving adaptive evolution. Apart from genetic variation, organisms also have other mechanisms that permit them to cope with

selective pressures by shaping their phenotypes in response to environmental changes. By doing so, organisms are able to shape (up to a certain point) their fitness potential to the new optimum. This capacity to generate different phenotypes under a similar genotypic background is called phenotypic plasticity (Figure 1.1), and can manifest in different ways: through changes in behaviour, developmental effects, by changing physiological responses, changing colours or modes of reproduction (Fusco & Minelli, 2010). The within-individual change inherent to plasticity increases the capacity for quicker responses to change, making plasticity likely to be a preferred mechanism for coping with environmental changes if adaptive genetic variation is not available. Specific examples of plasticity as being highly beneficial for responses to ongoing change include, for example, changes in insect developmental time and activity period under warmer environmental conditions leading to an increase in the number of generations per year (Mitton & Ferrenberg, 2012). This study on mountain pine beetles (*Dendroctonus ponderosae*) demonstrates how environmental change (in this case global warming) conferred these beetles with a higher adaptive potential to colonise previously inhospitable environments (higher latitudes and altitudes) and increased its reproductive success. Another example of a plastic response to environmental change is the case study of reproductive phenology in the great tit (*Parus major*), with reported shifts in mean egg-laying dates accompanying a corresponding increase in pre-laying temperatures over a period of five decades (Charmantier et al., 2008). Importantly, this shift accompanies a similar trend for emergence of a major source of food for these birds (caterpillar of the moth *Operophtera brumata*), which has enabled populations to maintain their fitness, something which is not happening in all populations. The change in egg-laying dates is likely to be based on phenotypic plasticity, since individuals breeding in consecutive years seem to be able to adjust to each year's temperature. Similar responses have been found in other organisms, showing the importance of taking phenology into account (Ponti & Sannolo, 2022; e.g., Carretero, 2006).

Despite these advantageous characteristics, plasticity can have potentially negative consequences on the long-term capacity for adaptation. An ongoing debate among evolutionary biologists is on whether plasticity reduces the efficacy of selection, since it leads to trait change without selection on the underlying genotype. A specific example of this phenomenon is given by the term “Bogert effect”, a concept in physiology that posits that evolutionary inertia of physiological traits arises when short-term behavioural responses are sufficient to respond to environmental stress, for example through microhabitat selection or changes in activity time (Muñoz et al., 2022). This effect has been demonstrated several times in ectothermic vertebrates that can, if needed,

successfully implement different thermoregulatory behaviours thus preventing natural selection from acting upon thermal physiology (Huey et al., 2003; Farallo et al., 2018). In some cases, plasticity can even be maladaptive, if it promotes a conflict between fitness components (for example, if survivorship is promoted at the cost of reproduction, Svensson et al., 2020). However, recent views suggest that plasticity and selection can act together to promote adaptation, since plasticity can serve as a rapid way for a population to “buy time” so that genetic variability can “catch up” to ensure long-term adaptation (Levis & Pfennig, 2016; Radersma et al., 2020). Such a case study is given by side-blotched lizards (*Uta stansburiana*) inhabiting dark-coloured substrates (Corl et al., 2018). Although colours of lizards living on and off darker lava flow habitats matched their environment (light for off-lava, dark for on-lava), switching substrates for 60 days led to changes in pigmentation to match the new substrate, implying phenotypic flexibility. However, these lizards cannot completely change their phenotype, suggesting underlying genetic variation differentiating dark and light lizards.

1.2. Altitudinal gradients as a means to explore organismal responses to environmental change

Altitudinal gradients, which can typically be found in mountain ranges, have long been of high interest for researchers in the study of ecological communities and biodiversity. In one of its many works, Carl Linnaeus (1744) wrote about elevational gradients and associated biological diversity, suggesting that higher diversity could be observed in habitats at higher elevations. In his book he was assuming a scenario in which the world had once been inundated, leaving only the highest elevations suitable for terrestrial life; species in these highland refugia would have later re-populated all habitats at lower elevations. Modern biogeographic models suggest that biodiversity frequently peaks at intermediate elevations (e.g. Brehm et al., 2007; Zhang et al., 2016) but a clear explanation for this is yet to be defined (Bertuzzo et al., 2016). Some examples suggest higher species richness and productivity at mid-altitude given higher amount of biomass at mid-altitudes, higher diversity in general or diversity in ecotones along the gradient, and higher extinction rates or lesser speciation events at the bottom or top of the altitudinal gradient (but see review in McCain & Grytnes, 2010). Despite the great complexities associated with studying altitudinal gradients, studies generally show an overall tendency for biodiversity to decrease in higher altitudes (Lomolino, 2001; Rahbek, 2005; Dreiss et al., 2015).

The way in which biotic communities are established along altitudinal gradients depends on how organisms respond to the environmental challenges associated with these environments. Altitudinal gradients have thus also been used as a means to explore the effects of environmental change because of the drastic changes in conditions that are typically found along altitude. Particularly at high altitudes (typically considered to start above 3,500 m above sea level, Mathew & Sharma, 2021) conditions are very challenging, characterized by very cold temperatures, high UV exposure and reduced oxygen availability due to low barometric pressure (Figure 1.2). Other environmental factors also frequently vary a lot relative to nearby lowland areas, such as habitat capacity, precipitation, anthropogenic pressure and geometric constraints (Körner, 2007; Nogués-Bravo et al., 2008). Ultimately, one must not forget that altitude is not a single factor to be studied in a simplistic manner, since it covaries with multiple environmental factors. Such conditions may create strong evolutionary pressures, which provide us with potential models to investigate adaptation in a changing world. In fact, geographic variation linked to altitude may provide a valuable proxy to investigate general patterns

of biological responses to environmental and climate change (e.g. Schneider & Mazer 2016), for example by studying adaptive phenotypes that arise in these conditions.

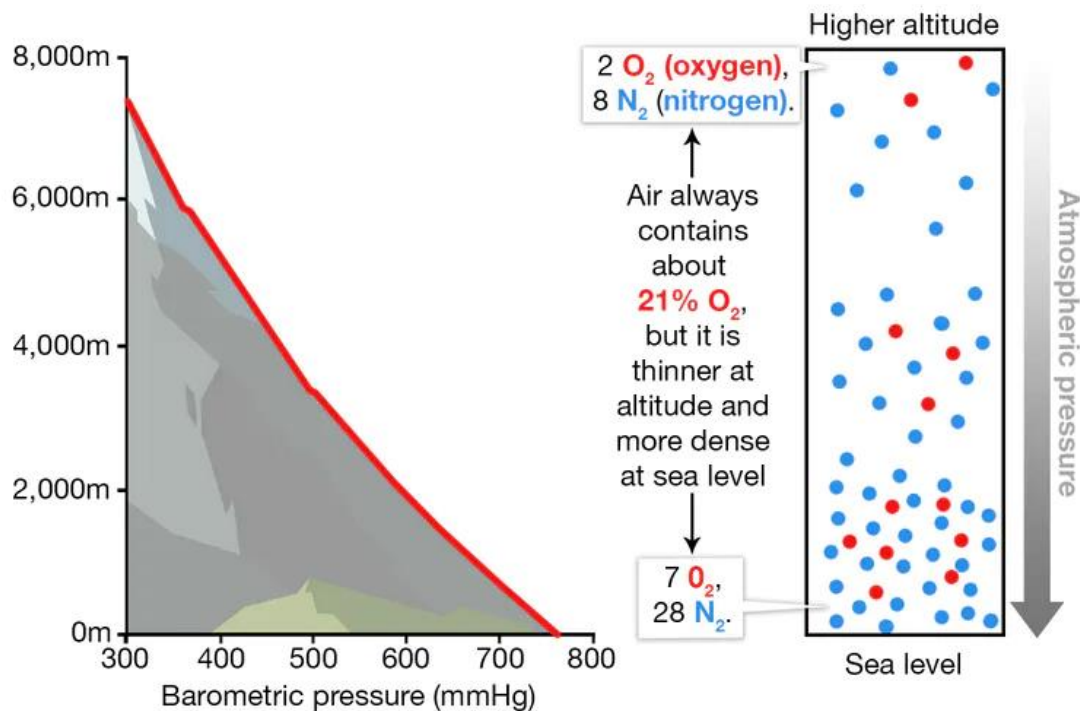


Figure 1.2. Increasing altitudes are associated with decreasing atmospheric oxygen pressure. Multiple studies have shown this to be one of the most important abiotic constraints for organisms living in high altitude habitats. Image by Brendan Scott (used under a CC-BY-ND license).

For example, some of the most widely studied suites of phenotypes changing with altitude are those linked to organismal physiology, and even knowledge on how these operate need to be integrated on a broader biological system framework. Animal flight at high altitudes is a good example. Flight already requires the use of large amounts of energy and oxygen under typical conditions, so its efficiency would be seriously compromised at high altitudes. This thus creates the necessity for the combined evolution of traits for flying animals adapted to altitude such as birds (reviewed by Scott 2011) or insects (reviewed by Dillon et al., 2006). As an example, bar-headed geese (*Anser indicus*) that migrate over the Tibetan plateau overcome these challenges with relatively enlarged lungs (Scott et al., 2011), higher ventilation efficiency (Scott & Milsom 2007), reduced metabolism (Meir et al., 2019) and higher affinity of hemoglobin to oxygen (Meir & Milsom, 2013). Similar traits are typically found in lineages that have evolved to adapt to high-altitude, but can also arise as within-individual plastic changes. For example, deer mice (*Peromyscus maniculatus*) populations from a common stock

translocated to field sites at high-altitude have been shown to develop larger organs (gut, lung and heart) and increased hematocrit (Hammond et al., 1999).

Recent advances in molecular biology and genomics (Stapley et al., 2010) have also allowed the possibility of understanding the roles of adaptation and acclimation to altitude, and which genes and metabolic pathways are expressed in specific tissues relevant to adaptation in these conditions. Examples of such genomic scans to understand adaptation to altitude have already been conducted in several organisms. These suggest multiple genomic paths to adaptation, either via simple genetic mechanisms, or through complex polygenic architectures. One such example showing how these different paths can occur even in closely related taxa are given by two studies on different species of bees that have contrasted genomic variation from highland and lowland populations. In one study with honey bees (*Apis mellifera*) from East Africa two large chromosomal inversions were found in highland populations, whereas these haplotypes were not found in other populations of honey bee worldwide (Wallberg et al., 2017). Genes within the inversions are hypothesized to be involved in behavioural traits such as foraging and learning, which are thought to be important for responding to highland conditions. In a different bee species from Asia (*Apis cerana*), signatures of positive selection in highland populations were found scattered throughout the whole genome, which suggests that multiple functions are crucial in adaptation (Montero-Mendieta et al., 2019). These and other population genomic studies contrasting populations at different altitudes provide a good opportunity to investigate these genomic mechanisms, although the potential for confounding genomic structure should always be considered.

Altitudinal genomics have been particularly advanced for mammalian model species. In several of these mammal models, despite strikingly different evolutionary histories and biology, signals of convergent evolution at the gene level have been found, particularly associated with adaptation to hypoxia via the *EPAS1* gene (endothelial PAS domain-containing protein 1). This gene is a transcription factor that is known to regulate organismal response to hypoxia. In human populations living in the Tibetan plateau, this locus is associated with signatures of both positive selection and introgression, specifically introgression from Denisovan hominins that are likely to have been adapted to high altitude environments (Huertasw4a-Sánchez et al., 2014). These Denisovan variants were later demonstrated to have been neutral in modern human populations long before positive selection, which occurred when the ancestors of modern Tibetan humans permanently colonized the Tibetan plateau after the last glacial maximum

(Zhang et al., 2021). For deer mice, another frequent model in studies of altitudinal adaptation, allelic variation in *EPAS1* was shown by Schweizer et al. (2019) to vary predictably with altitude. In particular, high altitude associated genotypes influenced both heart rate and transcriptomic responses under hypoxia regimes.

1.3. Adaptations to altitude in ectotherms, with a focus on lizards

Ectotherms are frequent model organisms in environmental change studies. These animals have a strong dependency on their surrounding environment to regulate their body temperature (Angilletta, 2009; Huey, 1982; Sinervo et al., 2010) which, in general, should make them more vulnerable to environmental changes than endotherms (Paaajmans et al., 2013). The extreme conditions of high altitude habitats can be particularly harmful, since temperature dramatically changes the physiological effects of reduced oxygen pressure (Sibly & Atkinson, 1994; Woods 1999; Portner, 2001). An example of this effect has been demonstrated for example in the fruit fly (*Drosophila melanogaster*): individuals that were raised under a low oxygen pressure regime and normal temperatures became 10-15% smaller than control individuals at normal oxygen pressure individuals; individuals reared under increasing temperatures at hypoxic conditions became 30-40% smaller (Frazier et al., 2001). Despite these limitations in some ectotherms, many ectothermic vertebrates appear to cope well with environmental changes and gradients, often showing a great capacity to respond (Seebacher et al., 2015; Crowell et al., 2021).

Lizards specifically are considered a key vertebrate group to study the effect of environmental changes impacts as they are ectotherms, sedentary, have relatively short-generation times and many species are ubiquitous (occupy a wide range of habitats) and can attain high densities. From a practical perspective they facilitate field monitoring and they are good models for experimental manipulation since lab husbandry is usually easy to pursue (Huey, 1982; Pianka & Vitt, 2003). Comparative ecophysiological studies in lizards are a topic of growing interest. In lizard ecophysiology, thermal or thermoregulatory studies can help to disentangle the adaptive or plastic component (or both) underlying responses to different climatic regimes, mediated by landscape structure (Sears & Angilletta, 2015), seasonal acclimation (Seebacher et al., 2003), reproductive state and body condition (Seebacher, 2005). Studies on thermal preferences and evaporative water loss rates may exhibit different patterns even if thermal physiology remains unaltered, reflecting shifts in precipitation regimes and hydric ecology (García-Muñoz & Carretero, 2013) in accordance with habitat and distribution range (Rato & Carretero, 2015). As altitude is a complex of environmental variables linked to steep variation in thermal, hydric, oxygen pressure and UV exposure parameters, different ecophysiology and behavioural traits should be tested in parallel to acknowledge these environmental effects of altitude on the physiology of lizards. While

ecophysiological neutrality either in the phylogeny or populations is commonly assumed, recent work demonstrates a non-parallel variation along altitudinal gradients between species (Žagar et al., 2015). Another recent study along an altitudinal gradient demonstrated that high altitude lizard populations are more resistant to water loss but high parasite loads at lower elevations can have an influence on how these physiological patterns are interpreted (Sannolo et al., 2020). As ectotherm species may suffer extreme conditions in a great altitudinal gradient we can assume stressing conditions can impact lizards living in such gradients consequently leading these organisms to change their thermal and hydric regulatory responses to maintain fitness and performance (Cowles & Bogert, 1944; Angilletta et al., 2002; Angilletta, 2009).

An ectotherm's environment shows a vital impact on its physiology, which then has natural consequences for its metabolism. Metabolism in ectotherms is thus highly reliant on factors such as temperature, humidity or oxygen pressure, and their complex interactions (Hicks & Wood 1985; Gillooly et al., 2001; Cardinale et al., 2002; Rozen-Rechels et al., 2019; Rubalcaba et al., 2020). Higher activity rates of antioxidant mechanisms are triggered to buffer ROS and prevent cell damage (Zamocky et al., 2008). Hence, metabolic assessments can highlight how physiological responses of lizards to altitude can be impacted by physiological stress (e.g. Seebacher et al., 2012; Žagar et al., 2015; Žagar et al., 2018). Specific impact on metabolism driven by one specific abiotic factor found in very high altitude (oxygen, namely low atmospheric oxygen pressure) was demonstrated to impact embryos of the common wall lizard (*Podarcis muralis*), where hypoxia (insufficient oxygen availability at tissue level) even at short periods of time disrupted normal embryo metabolism, evidenced by decreased heart rate and temporary decrease in carbon dioxide production (Cordero et al., 2017). Another example showing how phenotypic plasticity can underlie changes in metabolism and behaviour in high altitude was given by Qi et al. (2022). These authors translocated *Agama* spp. from low to high-altitudes and detected higher rates of fatty acid metabolism and storage in acclimatised lizards. Despite these indications of metabolic stress in high-altitude lizards, it is still unclear if this is reflected in lower survivability, which is a complex question due to many other confounding pressures (such as predation pressure, e.g. Martín & López, 1996). Studies on whether thermal physiology is impacted by hypoxia have obtained mixed results (Camacho et al., 2018; Díaz de la Vega-Pérez et al., 2019; Jiang et al., 2021; Bodensteiner et al., 2021).

Genetic and transcriptomic studies focusing on ectotherms living in high altitude areas have given further insights into how adaptive changes mediate adaptation in this

group, but this area of research is still in its infancy. Before the advent of large-scale genomic resources and low-cost sequencing, scans for loci associated with adaptation to environmental stress were based on candidate gene approaches or techniques with low genomic resolution. For example, Bonin et al. (2005) explored allelic variation in 392 amplified fragment length polymorphisms in the common frog (*Rana temporaria*) to detect associations to an altitudinal gradient. This approach identified some outlier loci with a potential association to elevation; however the absence of a properly assembled genome and corresponding annotation prevented the identification of the specific genes or genomic features that may explain the functional significance of these findings. Subsequent technological and analytical advances, mainly the development of genome-wide association studies or selective sweep mapping approaches, combined with the assembly and annotation of highly complete reference genomes, have opened the promise of identifying genetic changes associated with adaptation, if applied to suitable model systems. These state of the art approaches, however, are generally still lacking for ectotherms. An alternative approach has already been attempted for ectothermic vertebrates, based on comparing genomic sequences between highly divergent taxa to infer gene family evolution and signatures of positive selection with a potential link to altitudinal adaptation (Sun et al., 2018, Li et al., 2018). These studies suggest a role for genes involved with DNA repair, energy metabolism and response to hypoxia, but care should be taken when interpreting the obtained results, given that in these studies the organisms being compared typically show high evolutionary divergence. An additional source of evidence to better comprehend the molecular regulation of altitudinal adaptation is to conduct transcriptomic studies. While these techniques do not allow for the identification of genetic changes, since within-individual transcriptomic patterns are tissue and context dependent, these are still informative as to what genes may be differentially expressed when an organism is placed in these environments. For example, studies on high altitude frogs (Yang et al., 2016, Yang et al., 2017) have not found evidence for a role of genes associated with hypoxia response, with the authors suggesting that for these animals this response is not as necessary as what has been found for endotherms. Faced with these uncertainties, it is clear that suitable model organisms are needed to better understand how ectotherms cope with adaptation to high altitude.

1.4. *Gallotia galloti*, an endemic insular ectotherm that inhabits a steep altitudinal gradient in Tenerife (Canary Islands)

Oceanic islands are recognized as important systems to study evolutionary and ecological processes, with classic insights derived from patterns of biological diversity and functional evolution studies in insular communities (Gillespie, 2007; Warren et al., 2015). The way islands are: 1) elevated and eroded; 2) split and joined; 3) related to other islands and mainland together; and 4) characterized with their particular environmental features (attributed to geological, topographical and ecological factors that differ markedly from continental habitats) drive phenotypic evolution in particular and often predictable directions (Lomolino, 2005; Covas, 2012; Novosolov et al., 2013; Doutrelant et al., 2016). Most oceanic islands derive from volcanic activity acting throughout an extended period of geologic time, thus creating systems of closely located islands which, when colonized, can facilitate the occurrence of adaptive radiations. The classic example of this is the radiation in finch diversity in the Galapagos Islands, in which the diversification into multiple species following islands colonization was accompanied by high phenotypic diversification (Grant & Grant, 2003; Lamichhaney et al., 2015). Like the Galapagos, studies across many island systems worldwide have served to inform general hypothesis in biology. One of these oceanic island groups is the Canary Islands archipelago in the Atlantic, consisting of seven main islands, from west to east: El Hierro, La Palma, La Gomera, Tenerife, Gran Canaria, Fuerteventura and Lanzarote (Bowler, 2018). This archipelago is typically considered to be part of the Macaronesia biogeographic region, together with three other Atlantic archipelagos, those of Madeira, Azores and Cape Verde. Climate in the Canary Islands is generally stable, and mostly arid to temperate, owing to the low latitude and oceanic influence, although the large geographic variability creates the opportunity for multiple microclimates to arise (Bechtel, 2016). For example, the summit of the Teide volcano, in the island of Tenerife (3,718 metres above sea level) experiences a cold arid climate, with frequent snow cover in winter, and subalpine scrub as the main vegetation cover. The multiple islands and complex climate dynamics promoted multiple instances of evolutionary radiations, such as those of the plants of the genera *Micromeria* (Puppo et al., 2015; Curto et al., 2018), *Cheirolophus* (Vitales et al., 2014) and *Sonchus* (Kim et al., 1996).

Despite their relatively low dispersal ability, terrestrial reptiles have colonized the Canary Islands, including several species of *Tarentola* (Gekkonidae), *Chalcides*

(Scincidae) and *Gallotia* (Lacertidae), among others that are now extinct such as tortoises and snakes (López-Jurado & Mateo, 1993). Lizards from the genus *Gallotia*, Boulenger, 1916 (Squamata: Lacertidae: Gallotinae) are endemic to this archipelago, Figure 1.3, resulting from a single colonization event, likely 17-20 million years ago (Cox et al., 2010; sharing a common ancestor with *Psammodromus* from North Africa and Iberia). From this colonization event, several lineages have dispersed into the different islands of the archipelago resulting in what are typically recognized as seven species (excluding known extinct *Gallotia* species) from two main clades (Figure 1.3): one comprising *G. stehlini* (Gran Canaria), *G. atlantica* (Fuerteventura, Lanzarote), *G. simonyi* (El Hierro), *G. bravoana* (La Gomera) and *G. intermedia* (Tenerife), and a second clade with *G. caesaris* (La Gomera and El Hierro) and *G. galloti* (La Palma and Tenerife) (González et al., 1996; Hernández et al., 2001; Arnold et al., 2007, Cox et al., 2010). Using data from four mitochondrial loci, Cox et al. (2010) reconstituted the colonization history of the several *Gallotia* lineages in the Canary Islands, suggesting a complex stepping stone model that started on the easternmost islands (closer to mainland Africa), with further colonization events dependent on the geological formation of the islands. This biological radiation in a group of volcanic islands with well-known geological history and microclimatic variability offers an excellent opportunity to investigate patterns of adaptation and biological diversification, in particular associated with the response to environmental change.

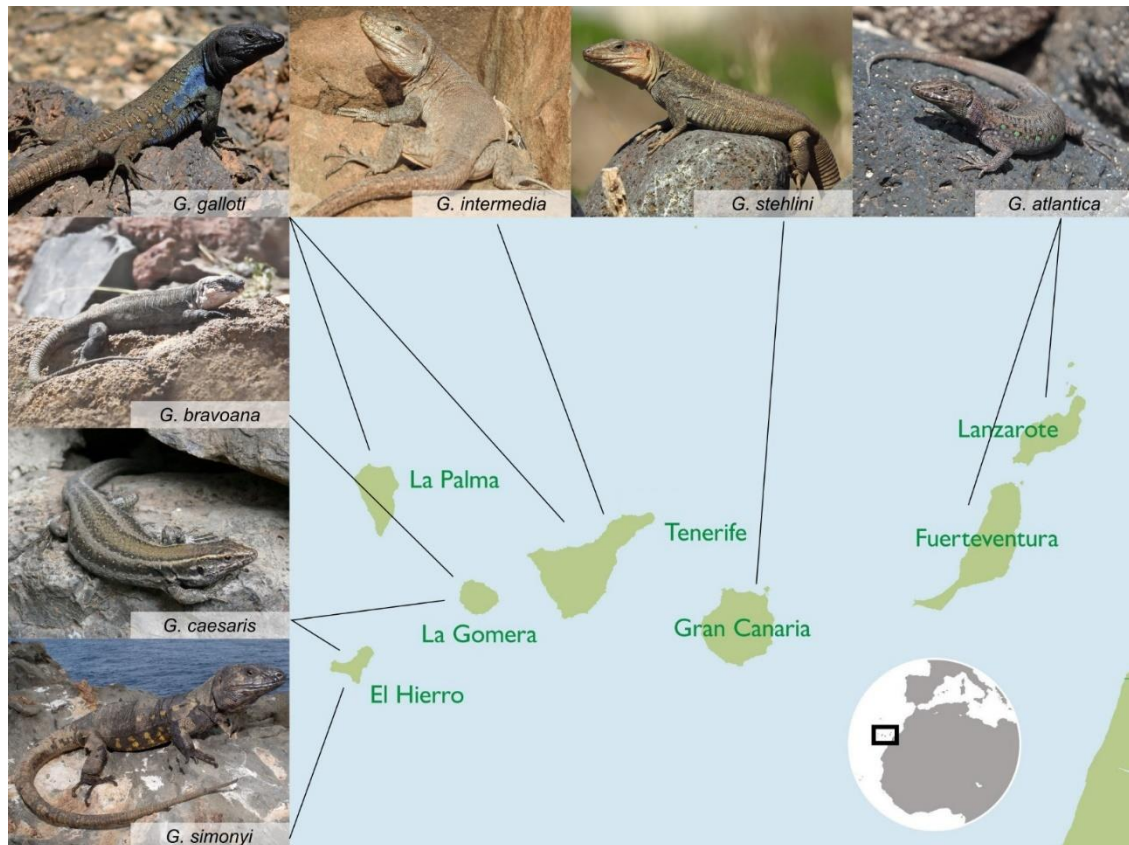


Figure 1.3. Map of the Canary Islands archipelago and the seven extant *Gallotia* species. Photo credits: *G. atlantica* (Sébastien Sant, CC-BY-NC 4.0); *G. stehlini* (Siegfried Troidl, CC-BY-NC 4.0); *G. intermedia* (Siegfried Troidl, CC-BY-NC 4.0); *G. galloti* (Siegfried Troidl, CC-BY-NC 4.0); *G. bravoana* (Cristoph Moning, CC-BY 4.0); *G. caesaris* (Haplochromis); *G. simonyi* (Domingo Trujillo González).

Among *Gallotia* species, *G. galloti* is a particularly suitable study system to pursue this line of research, particularly its lineages that occur in Tenerife because of its highest altitudinal gradient in the archipelago. Moreover, Tenerife combines several abiotic and biotic characteristics that make it highly heterogeneous. Its paleogeographic history is linked to volcanic activity around 2-4 Mya, during the late Miocene–Pliocene, which led to the secondary connection of three paleoislands into the current island we know today (Ancochea et al., 1990). Even today, the three massifs that once were paleoislands bare distinct geological and geomorphological characteristics than the younger central part, which includes both the Las Cañadas caldera and the Teide volcano cone that had his last eruptive episode circa 30,000 years ago (Carracedo et al., 2007). *G. galloti* is thought to have colonized the paleoislands of Tenerife between 2 to 4 Mya (Cox et al., 2010), and has since diverged into three lineages whose evolutionary history was likely shaped by the island's volcanic history, topography and microclimates (Thorpe & Brown, 1989). Specifically, one lineage is restricted to the northeastern Roque

de Fuera de Anaga islet (*G. g. insulanagae*), another lineage is found in the island's north side (*G. g. eisentrauti*) and a third one is distributed throughout the island's southern side (*G. g. galloti*), this last one occupying habitats along a very steep altitudinal gradient, from sea level up to the summit of the Teide (Fariña et al., 2011). A study performed by Thorpe & Brown (1989) focusing on the distribution of colour pattern patches along many different sampling locations indicated that the major source of differentiation occurred between lizards sampled on the north or south side of the island, broadly concordant to the current taxonomic classification into *G. g. eisentrauti* and *G. g. galloti* (Figure 1.4). An additional work made by the same authors showed that size (snout-vent length) of adult males tended to decrease with altitude (Thorpe & Brown, 1991), but this study did not differentiate between lineages so interpretation can be misleading, since *G. g. eisentrauti* does not attain the same high altitudes as *G. g. gallotia*. Genetic analyses of these populations have yet to yield a definitive answer on the evolutionary history and patterns of gene flow between these lineages, although there are some indications of gene flow between subspecies within the island (Richard & Thorpe, 2001; Thorpe & Richard, 2001; Brown et al., 2016).



Figure 1.4. The two main lineages of *Gallotia galloti* that can be found in Tenerife: *G. g. eisenrauti* and *G. g. galloti*. Dashed line on the map of Tenerife signals the approximate delimitation of their distribution within the island. Below, landscape of Tenerife in the southern part of the island (drier) showing the Teide volcano in the background. Credits for photos: *G. g. eisenrauti* (Jan Ebr, CC-BY 4.0); *G. g. galloti* (Siegfried Troidl, CC-BY-NC 4.0); Tenerife (Ingo Mehling).

Studies on how these lizards cope with environmental variation, particularly that associated with altitude, are scarce. Fariña et al. (2011) characterized the higher altitude populations and confirmed they belong to *G. g. galloti*, indicating other aspects of their biology such as reduced detectability (despite activity) during colder sampling periods and a generalist and opportunistic diet. A recent ecophysiological study published in 2022 evaluated patterns of thermal preferences and evaporative water loss in some populations on the island at different altitudes, and found differences in water loss rates but not in thermal preferences (Albaladejo-Robles et al., 2022). These authors however did not take into account potential evolutionary effects (populations of *G. g. eisenrauti* differ in their habitat preferences compared to the southern *G. g. galloti*, see for example Thorpe & Brown, 1989).

Thus, although studies on ecology and evolution of Canarian lizards were conducted in the past, further effort is needed to understand how these lizards cope with the extreme environmental gradients found in their distribution. Secondly, the high diversity and complexity of environmental conditions across the altitude can serve as a proxy for testing local adaptation and plasticity using *G. galloti* as a perfect model species. Moreover, several characteristics make this species a very good (but still underutilized) model for evolutionary and ecological studies: they are abundant, easy to capture, are resistant to experimental manipulation, lack predators or species occupying a similar niche (thereby reducing confounding effects of intra or interspecific interactions) and seem to have a large tolerance for environmental variation.

1.5. Objectives and organization of the thesis

In this thesis I use an integrative approach to assess the evolutionary and plastic responses to naturally occurring environmental variation across an extreme altitude gradient (~3,700 m) by using *G. galloti*, an endemic species to the Canary Islands archipelago, as a model species. This species is widely distributed within Tenerife, occupying habitats with a large range of environmental conditions within a relatively small geographic range, including high microclimatic and altitudinal variation. As specific objectives, I propose to:

- 1) Infer the degree of flexibility in the fundamental niche analysing several metabolic, ecophysiological and biochemical indicators;
- 2) Assess the mechanisms for phenotypic plasticity in terms of variation in size and longevity;
- 3) Conduct genomic scans to identify signatures of natural selection associated with high altitude populations, based on a *de novo* reference genome assembly and annotation for *G. galloti*;
- 4) Determine the effects of long-term evolution versus short-term adaptation in the system by comparing metabolism and ecophysiology in multiple populations of two lizard lineages;
- 5) Overall, evaluate the interplay between phenotypic plasticity and adaptation in response to high altitudes in a widespread insular lizard.

The thesis is composed of five chapters, which include three novel research studies focusing on the life history, physiology, metabolism and genomics of *G. galloti*. Chapter I consists of a general introduction of the main topics covered in the thesis. In Chapter II we focus on how lizards cope with environmental stress along a steep altitudinal gradient. We employ a variety of methods to study age structure, thermoregulatory and hydric physiology, metabolism and biochemical signatures of stress to understand the response profiles of different populations. Chapter III investigates the genetic basis of adaptation to high altitude in this species by means of a genomic approach. For this, we first perform an assembly and annotation of the

genome of *G. g. galloti*. We then re-sequence pools of populations at different altitudes and conduct whole-genome scans for signatures of selection. In Chapter IV we compare two *G. galloti* lineages living in Tenerife (*G. g. galloti* and *G. g. eisentrauti*) to understand how variation in ecophysiology and biochemical stress could have shaped the evolution of these two evolutionary units, which inhabit very different climatic and environmental conditions in opposing sides of the same mountain of a single island. Finally, Chapter V is an extended discussion of the main findings from the previous chapters, and we delineate guidelines for subsequent work.

1.6. References

Albaladejo-Robles, G., Rodríguez, N., Rodríguez-Concepción, B., Nogales, M., & Vences, M. (2022). Limited ecophysiological variation in *Gallotia galloti* (Oudart, 1839) lizards across an elevational gradient of over 3,500 meters (Squamata: Lacertidae). *Herpetology Notes*, 15, 87-96.

Ancochea, E., Fuster, J., Ibarrola, E., Cendrero, A., Coello, J., Hernan, F., Cantagrel, M. & Jamond, C. (1990). Volcanic evolution of the island of Tenerife (Canary Islands) in the light of new K-Ar data. *Journal of Volcanology and Geothermal Research*, 44(3-4), 231-249.

Angilletta MJ, Niewiarowski PH, Navas CA. (2002) The evolution of thermal physiology in ectotherms. *Journal of thermal biology*; 27, 249–268.

Angilletta MJ. Thermal Adaptation: A Theoretical and Empirical Synthesis. (2009). Oxford University Press Inc New York, USA.

Areta, J. I., Salvador, S. A., Gandoy, F. A., Bridge, E. S., Gorleri, F. C., Pegan, T. M., Gulson-Castillo, E. R., Hobson, K. A., & Winkler, D. W. (2021). Rapid adjustments of migration and life history in hemisphere-switching cliff swallows. *Current Biology*, 31(13), 2914-2919.

Arnold, E. N., Arribas, O., & Carranza, S. (2007). Systematics of the Palaearctic and Oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. *Zootaxa*, 1430(1), 1-86.

Barnosky, A. D., Matzke, N., Tomiya, S., Wogan, G. O., Swartz, B., Quental, T. B., Marshall, C., McGuire, J. L., Lindsey, E. L., Maguire, K. C., Mersey, B & Ferrer, E. A. (2011). Has the Earth's sixth mass extinction already arrived?. *Nature*, 471(7336), 51-57.

Barrett, R. D., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in ecology & evolution*, 23(1), 38-44.

Bechtel, B. (2016). The climate of the Canary Islands by annual cycle parameters. Int. Arch. Photogramm. Remote Sens. *Journal of Spatial Information Science*, XLI-B8, 243–250.

- Behm, J. E., & Kiers, E. T. (2014). A phenotypic plasticity framework for assessing intraspecific variation in arbuscular mycorrhizal fungal traits. *Journal of Ecology*, 102(2), 315-327.
- Bertuzzo, E., Carrara, F., Mari, L., Altermatt, F., Rodriguez-Iturbe, I., & Rinaldo, A. (2016). Geomorphic controls on elevational gradients of species richness. *Proceedings of the National Academy of Sciences*, 113(7), 1737-1742.
- Bodensteiner, B. L., Gangloff, E. J., Kouyoumdjian, L., Muñoz, M. M., & Aubret, F. (2021). Thermal–metabolic phenotypes of the lizard *Podarcis muralis* differ across elevation, but converge in high-elevation hypoxia. *Journal of Experimental Biology*, 224(24), jeb243660.
- Bonin, A., Taberlet, P., Miaud, C., & Pompanon, F. (2005). Explorative genome scan to detect candidate loci for adaptation along a gradient of altitude in the common frog (*Rana temporaria*). *Molecular biology and evolution*, 23(4), 773-783.
- Bowler, J. (2018). *Wildlife of Madeira and the Canary Islands: A Photographic Field Guide to Birds, Mammals, Reptiles, Amphibians, Butterflies and Dragonflies*. Princeton University Press.
- Brehm, G., Colwell, R. K., & Kluge, J. (2007). The role of environment and mid-domain effect on moth species richness along a tropical elevational gradient. *Global Ecology and Biogeography*, 16(2), 205-219.
- Brown, R. P., Paterson, S., & Risse, J. (2016). Genomic signatures of historical allopatry and ecological divergence in an island lizard. *Genome Biology and Evolution*, 8(11), 3618-3626.
- Camacho, A., Vandenbrooks, J. M., Riley, J., Telemeco, R. S. and Angilletta, M. J. jr. (2018). Oxygen supply did not affect how lizards responded to thermal stress. *Integrative Zoology*, 13, 428-436.
- Cardinale, B. J., Palmer, M. A., Swan, C. M., Brooks, S., & Poff, N. L. (2002). The influence of substrate heterogeneity on biofilm metabolism in a stream ecosystem. *Ecology*, 83(2), 412-422.

Carracedo, J. C., Badiola, E. R., Guillou, H., Paterne, M., Scaillet, S., Torrado, F. Paris, R., Fra-Paleo, U. and Hansen, A. (2007). Eruptive and structural history of Teide Volcano and rift zones of Tenerife, Canary Islands. *Geological Society of America Bulletin*, 119(9-10), 1027-1051.

Carretero, M. A. (2006): Reproductive cycles in Mediterranean lacertids: Plasticity and constraints. pp. 33-54, in: Corti, C.; Lo Cascio, P. & Biaggini M.(eds.) Mainland and insular lacertid lizards: a Mediterranean perspective. Firenze University Press. Florence.

Catullo, R. A., Llewelyn, J., Phillips, B. L., & Moritz, C. C. (2019). The potential for rapid evolution under anthropogenic climate change. *Current Biology*, 29(19), R996-R1007.

Ceballos, G., Ehrlich, P. R., & Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proceedings of the National Academy of Sciences*, 114(30), E6089-E6096.

Ceballos, G., Ehrlich, P. R., & Raven, P. H. (2020). Vertebrates on the brink as indicators of biological annihilation and the sixth mass extinction. *Proceedings of the National Academy of Sciences*, 117(24), 13596-13602.

Charmantier, A., McCleery, R. H., Cole, L. R., Perrins, C., Kruuk, L. E. B., & Sheldon, B. C. (2008). Adaptive Phenotypic Plasticity in Response to Climate Change in a Wild Bird Population. *Science*, 320(5877), 800–803.

Colwell, R. K., & Lees, D. C. (2000). The mid-domain effect: geometric constraints on the geography of species richness. *Trends in ecology & evolution*, 15(2), 70-76.

Cordero, G. A., Andersson, B. A., Souchet, J., Micheli, G., Noble, D. W., Gangloff, E. J., Uller, T. & Aubret, F. (2017). Physiological plasticity in lizard embryos exposed to high-altitude hypoxia. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 327(7), 423-432.

Corl, A., Bi, K., Luke, C., Challa, A. S., Stern, A. J., Sinervo, B., & Nielsen, R. (2018). The genetic basis of adaptation following plastic changes in coloration in a novel environment. *Current Biology*, 28(18), 2970-2977.

Covas, R. (2012). Evolution of reproductive life histories in island birds worldwide. *Proceedings of the Royal Society B: Biological Sciences*, 279(1733), 1531-1537.

Cowie, R. H., Bouchet, P., & Fontaine, B. (2022). The Sixth Mass Extinction: fact, fiction or speculation? *Biological Reviews*, 97, 640-663.

Cowles R. B., & Bogert C. M. (1944). A preliminary study of the thermal requirements of desert reptiles. *Bulletin of the AMNH*; v. 83, article 5

Cox, S. C., Carranza, S., & Brown, R. P. (2010). Divergence times and colonization of the Canary Islands by *Gallotia* lizards. *Molecular Phylogenetics and Evolution*, 56(2), 747-757.

Crowell, H. L., King, K. C., Whelan, J. M., Harmel, M. V., Garcia, G., Gonzales, S. G., Maier, P. H., NeldnerH., Nhu, T., Nolan, J. T., & Taylor, E. N. (2021). Thermal ecology and baseline energetic requirements of a large-bodied ectotherm suggest resilience to climate change. *Ecology and Evolution*, 11(12), 8170-8182.

Curto, M., Schachtler, C., Puppo, P., & Meimberg, H. (2018). Using a new RAD-sequencing approach to study the evolution of *Micromeria* in the Canary Islands. *Molecular Phylogenetics and Evolution*, 119, 160-169.

Davis, M. B., & Shaw, R. G. (2001). Range shifts and adaptive responses to Quaternary climate change. *Science*, 292(5517), 673-679.

Davis, M. B., Shaw, R.G., & Etterson, J.R. (2005). Evolutionary responses to changing climate. *Ecology* 86, 1704-1714.

Díaz de la Vega-Pérez, A. H., Barrios-Montiel, R., Jiménez-Arcos, V. H., Bautista, A., & Bastiaans, E. (2019). High-mountain altitudinal gradient influences thermal ecology of the Mesquite Lizard (*Sceloporus grammicus*). *Canadian Journal of Zoology*, 97(8), 659-668.

Dillon, M. E., Frazier, M. R., & Dudley, R. (2006). Into thin air: physiology and evolution of alpine insects. *Integrative and Comparative Biology*, 46(1), 49-61.

Doutrelant, C., Paquet, M., Renoult, J. P., Grégoire, A., Crochet, P. A., & Covas, R. (2016). Worldwide patterns of bird colouration on islands. *Ecology Letters*, 19(5), 537-545.

Dreiss, L. M., Burgio, K. R., Cisneros, L. M., Klingbeil, B. T., Patterson, B. D., Presley, S. J., & Willig, M. R. (2015). Taxonomic, functional, and phylogenetic dimensions of rodent biodiversity along an extensive tropical elevational gradient. *Ecography*, 38(9), 876-888.

Duraiappah, A. K., Naeem, S., Agardy, T., Ash, N. J., Cooper, H. D., Díaz, S., Faith, D. P., Mace, G., McNeely, J. A., Mooney, H. A., et al.. (2005). Ecosystems and human well-being: biodiversity synthesis; a report of the Millennium Ecosystem Assessment.

Elbers, A. R. W., Koenraadt, C. J., & Meiswinkel, R. (2015). Mosquitoes and Culicoides biting midges: vector range and the influence of climate change. *Revue Scientifique et Technique*, 34(1), 123-37.

Farallo, V. R., Wier, R., & Miles, D. B. (2018). The Bogert effect revisited: Salamander regulatory behaviors are differently constrained by time and space. *Ecology and Evolution*, 8(23), 11522-11532.

Fariña, B., Navarro, M. L. R., López, M. R., & Moreno, J. E. (2011). Sobre la presencia de Gallotia galloti en el pico del volcán Teide (Tenerife, Islas Canarias). *Boletín de la Asociación Herpetológica Española*, (22), 46-51.

Frazier, M. R., Woods, H. A., & Harrison, J. F. (2001). Interactive effects of rearing temperature and oxygen on the development of *Drosophila melanogaster*. *Physiological and Biochemical Zoology*, 74(5), 641-650.

Fusco, G., & Minelli, A. (2010). Phenotypic plasticity in development and evolution: facts and concepts. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365(1540), 547-556.

García-Muñoz, E., & Carretero, M. A. (2013). Comparative ecophysiology of two sympatric lizards. Laying the groundwork for mechanistic distribution models. *Acta Herpetologica*, 8(2), 123-128.

Gillespie, R. G. (2007). Oceanic islands: models of diversity. *Encyclopedia of biodiversity*, 1-13.

Gillooly, J. F., Brown, J. H., West, G. B., Savage, V. M., & Charnov, E. L. (2001). Effects of size and temperature on metabolic rate. *Science*, 293(5538), 2248-2251.

González, P., Pinto, F., Nogales, M., Jiménez-Asensio, J., Hernández, M., & Cabrera, V. M. (1996). Phylogenetic Relationships of the Canary Islands Endemic Lizard Genus *Gallotia* (Sauria: Lacertidae), Inferred from Mitochondrial DNA Sequences. *Molecular phylogenetics and evolution*, 6(1), 63-71.

Grant, R. B., & Grant, P. R. (2003). What Darwin's finches can teach us about the

evolutionary origin and regulation of biodiversity. *BioScience*, 53(10), 965-975.

Hammond, K. A., Roth, J., Janes, D. N., & Dohm, M. R. (1999). Morphological and physiological responses to altitude in deer mice *Peromyscus maniculatus*. *Physiological and Biochemical Zoology*, 72(5), 613-622.

Hernández, M., Macameyer, N., Rando, J. C., Valido, A., & Nogales, M. (2001). Addition of a new living giant lizard from La Gomera island to the phylogeny of the endemic genus *Gallotia* (Canarian archipelago). *HERPETOLOGICAL JOURNAL*, 11, pp. 171-173.

Hicks, J. W., & Wood, S. C. (1985). Temperature regulation in lizards: effects of hypoxia. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 248(5), R595-R600.

Hof, A. E., Campagne, P., Rigden, D. J., Yung, C. J., Lingley, J., Quail, M. A., Hall, N., Darby, A. C. & Saccheri, I. J. (2016). The industrial melanism mutation in British peppered moths is a transposable element. *Nature*, 534(7605), 102-105.

Huerta-Sánchez, E., Jin, X., Bianba, Z., Peter, B. M., Vinckenbosch, N., Liang, Y., Yi, X., He, M., Somel, M., Ni, P., Wang, B., Ou, X., H., Luosang, J., Xi, Z., Cuo, P., Li, K., Gao, G., Yin, Y., Wang, W., Zhang, X., Xu, X., Yang, H. & Nielsen, R. (2014). Altitude adaptation in Tibetans caused by introgression of Denisovan-like DNA. *Nature*, 512(7513), 194-197.

Huey, R.B. (1982). Temperature, physiology, and the ecology of reptiles, in: C. Gans, F.H. Pough (Eds.), *Biology of the Reptilia* 12. Academic Press, 25-91.

Huey, R. B., Hertz, P. E., & Sinervo, B. (2003). Behavioral drive versus behavioral inertia in evolution: a null model approach. *The American Naturalist*, 161(3), 357-366.

IUCN (2021). The IUCN Red List of Threatened Species. Version 2021-2. <https://www.iucnredlist.org>. Downloaded on March 2022.

Jiang, Z. W., Ma, L., Mi, C. R., & Du, W. G. (2021). Effects of hypoxia on the thermal physiology of a high-elevation lizard: implications for upslope-shifting species. *Biology Letters*, 17(3), 20200873.

Jones, M. R., Mills, L. S., Alves, P. C., Callahan, C. M., Alves, J. M., Lafferty, D. J., Jiggins, F. M., Jensen, J. F., Melo-Ferreira, J. & Good, J. M. (2018). Adaptive

introgression underlies polymorphic seasonal camouflage in snowshoe hares. *Science*, 360(6395), 1355-1358.

Jones, M. R., Mills, L. S., Jensen, J. D., & Good, J. M. (2020). Convergent evolution of seasonal camouflage in response to reduced snow cover across the snowshoe hare range. *Evolution*, 74(9), 2033-2045.

Kim, S. C., Crawford, D. J., Francisco-Ortega, J., & Santos-Guerra, A. (1996). A common origin for woody *Sonchus* and five related genera in the Macaronesian islands: molecular evidence for extensive radiation. *Proceedings of the National Academy of Sciences*, 93(15), 7743-7748.

Körner, C. (2007). The use of 'altitude' in ecological research. *Trends in ecology & evolution*, 22(11), 569-574.

Lamichhaney, S., Berglund, J., Almén, M. S., Maqbool, K., Grabherr, M., Martinez-Barrio, A., Promerová, M., Rubin, C-J, Wang, C., Zamani, N., Grant, B. R., Grant, P. R., Webster, M. T. & Andersson, L. (2015). Evolution of Darwin's finches and their beaks revealed by genome sequencing. *Nature*, 518(7539), 371-375.

Le Roux, J. J., Hui, C., Castillo, M. L., Iriondo, J. M., Keet, J. H., Khapugin, A. A., Médail, F., Rejmánek, M., Theron, G., Yannelli, F. A. & Hirsch, H. (2019). Recent anthropogenic plant extinctions differ in biodiversity hotspots and coldspots. *Current Biology*, 29(17), 2912-2918.

Levis, N. A., & Pfennig, D. W. (2016). Evaluating 'plasticity-first' evolution in nature: key criteria and empirical approaches. *Trends in ecology & evolution*, 31(7), 563-574.

Li, J. T., Gao, Y. D., Xie, L., Deng, C., Shi, P., Guan, M. L., Huang, S., Ren, J. L., Wu, D. D., Ding, L., Huang, Z. Y., Nie, H., Humphreys, D. P., Hillis, D. M., Wang, W. Z. & Zhang, Y. P. (2018). Comparative genomic investigation of high-elevation adaptation in ectothermic snakes. *Proceedings of the National Academy of Sciences*, 115(33), 8406-8411.

Lomolino, M. V. (2001). *Special Issue: Diversity Patterns in Small Mammals among Elevational Gradients || Elevational Gradients of Species-Density: Historical and Prospective Views. Global Ecology and Biogeography*, 10(1), 3-13.

- Lomolino, M. V. (2005). Body size evolution in insular vertebrates: generality of the island rule. *Journal of Biogeography*, 32(10), 1683-1699.
- López-Jurado, L. F., & Mateo, J. A. (1993). A new giant land tortoise from the Pliocene of Gran Canaria (Canary Islands). *Revista española de Herpetología*.
- MacLaurin, J., & Sterelny, K. (2008). What is biodiversity?. In *What Is Biodiversity?*. University of Chicago Press.
- Martín, J., & López, P. (1996). Avian predation on a large lizard (*Lacerta lepida*) found at low population densities in Mediterranean habitats: an analysis of bird diets. *Copeia*, 1996(3), 722-726.
- Mathew T. M. & Sharma S. (2021). High Altitude Oxygenation. [Updated 2022 Apr 14]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-.
- McCain, C. M., & Grytnes, J. A. (2010). Elevational gradients in species richness. *eLS*.
- Meir, J. U., & Milsom, W. K. (2013). High thermal sensitivity of blood enhances oxygen delivery in the high-flying bar-headed goose. *Journal of Experimental Biology*, 216(12), 2172-2175.
- Meir, J. U., York, J. M., Chua, B. A., Jardine, W., Hawkes, L. A., & Milsom, W. K. (2019). Reduced metabolism supports hypoxic flight in the high-flying bar-headed goose (*Anser indicus*). *Elife*, 8.
- Midgley, G. F., Hannah, L., Millar, D., Thuiller, W., & Booth, A. (2003). Developing regional and species-level assessments of climate change impacts on biodiversity in the Cape Floristic Region. *Biological conservation*, 112(1-2), 87-97.
- Millennium Ecosystem Assessment (2005): Ecosystems and Human Well-being: Synthesis. Island Press, Washington, DC.
<https://www.millenniumassessment.org/documents/document.356.aspx.pdf>
- Mitton, J. B., & Ferrenberg, S. M. (2012). Mountain pine beetle develops an unprecedented summer generation in response to climate warming. *The American Naturalist*, 179(5), E163-E171.

Montero-Mendieta, S., Tan, K., Christmas, M. J., Olsson, A., Vilà, C., Wallberg, A., & Webster, M. T. (2019). The genomic basis of adaptation to high-altitude habitats in the eastern honey bee (*Apis cerana*). *Molecular Ecology*, 28(4), 746-760.

Muñoz, M. M., Feeley, K. J., Martín, P. H. & Farallo, V. R. (2022). The multidimensional (and contrasting) effects of environmental warming on a group of montane tropical lizards. *Functional Ecology*, 36, 419-431

Nogués-Bravo, D., Araújo, M. B., Romdal, T., & Rahbek, C. (2008). Scale effects and human impact on the elevational species richness gradients. *Nature*, 453(7192), 216–219.

Noss, R. F. (1990). Indicators for Monitoring Biodiversity: A Hierarchical Approach. - *Conservation Biology*, 4, 355-364.

Novosolov, M., Raia, P., & Meiri, S. (2013). The island syndrome in lizards. *Global Ecology and Biogeography*, 22(2), 184-191.

Paaijmans, K. P., Heinig, R. L., Seliga, R. A., Blanford, J. I., Blanford, S., Murdock, C. C., & Thomas, M. B. (2013). Temperature variation makes ectotherms more sensitive to climate change. *Global change biology*, 19(8), 2373-2380.

Payne, J. L., Bush, A. M., Heim, N. A., Knope, M. L., & McCauley, D. J. (2016). Ecological selectivity of the emerging mass extinction in the oceans. *Science*, 353(6305), 1284-1286.

Perry, A. L., Low, P. J., Ellis, J. R., & Reynolds, J. D. (2005). Climate change and distribution shifts in marine fishes. *Science*, 308(5730), 1912-1915.

Pianka, E.R. & Vitt, L.J. (2003). Lizards: windows to the evolution of diversity. Univiversity of California Press, California.

Pimm, S. L., Jenkins, C. N., Abell, R., Brooks, T. M., Gittleman, J. L., Joppa, L. N., Raven, P. H., Roberts, C. M., & Sexton, J. O. (2014). The biodiversity of species and their rates of extinction, distribution, and protection. *Science*, 344(6187), 1246752.

Ponti, R. & Sannolo, M. (2022). The importance of including phenology when modelling species: ecological niche. *Ecography* e06143.

- Portner, H. O. (2001). Climate change and temperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* 88: 137–146.
- Puppo, P., Curto, M., Gusmão-Guedes, J., Cochofel, J., de Paz, P. L. P., Bräuchler, C., & Meimberg, H. (2015). Molecular phylogenetics of *Micromeria* (Lamiaceae) in the Canary Islands, diversification and inter-island colonization patterns inferred from nuclear genes. *Molecular Phylogenetics and Evolution*, 89, 160-170.
- Qi, Y., Zhang, T., Wu, Y., Yao, Z., Qiu, X., Tang, X., Fu, J. & Yang, W. (2022). A multi-level assessment of plasticity in response to high-altitude environment for agama lizards. *Frontiers in Ecology and Evolution*, 178.
- Radersma, R., Noble, D. W., & Uller, T. (2020). Plasticity leaves a phenotypic signature during local adaptation. *Evolution Letters*, 4(4), 360-370
- Rahbek, C. (2005). The role of spatial scale and the perception of large-scale species-richness patterns. *Ecology letters*, 8(2), 224-239.
- Rato, C., & Carretero, M. A. (2015). Ecophysiology tracks phylogeny and meets ecological models in an Iberian gecko. *Physiological and Biochemical Zoology*, 88(5), 564-575.
- Richard, M., & Thorpe, R. S. (2001). Can microsatellites be used to infer phylogenies? Evidence from population affinities of the Western Canary Island lizard (*Gallotia galloti*). *Molecular Phylogenetics and Evolution*, 20(3), 351-360.
- Rozen-Rechels, D., Dupoué, A., Lourdaïs, O., Chamaillé-Jammes, S., Meylan, S., & Clobert, J. (2019). When water interacts with temperature: ecological and evolutionary implications of thermo-hydreregulation in terrestrial ectotherms. *Ecology and Evolution*, 9(17), 10029-43.
- Rubalcaba, J. G., Verberk, W. C., Hendriks, A. J., Saris, B., & Woods, H. A. (2020). Oxygen limitation may affect the temperature and size dependence of metabolism in aquatic ectotherms. *Proceedings of the National Academy of Sciences*, 117(50), 31963-31968.
- Sannolo, M., Civantos, E., Martín, J. & Carretero, M. A. (2020): Variation in field body temperature and resistance to dehydration along an environmental gradient in a diurnal ectotherm. *Journal of Zoology*, 310 221-231

Schachat, S. R., & Labandeira, C. C. (2021). Are insects heading toward their first mass extinction? Distinguishing turnover from crises in their fossil record. *Annals of the Entomological Society of America*, 114(2), 99-118.

Schneider, H. E., & Mazer, S. J. (2016). Geographic variation in climate as a proxy for climate change: Forecasting evolutionary trajectories from species differentiation and genetic correlations. *American Journal of Botany*, 103(1), 140-152.

Schweizer, R. M., Velotta, J. P., Ivy, C. M., Jones, M. R., Muir, S. M., Bradburd, G. S., Storz, J. F., Scott, G. R. & Cheviron, Z. A. (2019). Physiological and genomic evidence that selection on the transcription factor *Epas1* has altered cardiovascular function in high-altitude deer mice. *PLoS genetics*, 15(11), e1008420.

Scott, G. R., & Milsom, W. K. (2007). Control of breathing and adaptation to high altitude in the bar-headed goose. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 293(1), R379-R391.

Scott, G. R. (2011). Elevated performance: the unique physiology of birds that fly at high altitudes. *Journal of Experimental Biology*, 214(15), 2455-2462.

Scott, G. R., Schulte, P. M., Egginton, S., Scott, A. L. M., Richards, J. G. and Milsom, W. K. (2011). Molecular evolution of cytochrome c oxidase underlies high altitude adaptation in the bar-headed goose. *Molecular Biology and Evolution* 28, 351-363.

Sears, M.W., Angilletta Jr, M.J. (2015). Costs and benefits of thermoregulation revisited: both the heterogeneity and spatial structure of temperature drive energetic costs,

Seebacher, F. (2005). A review of thermoregulation and physiological performance in reptiles: what is the role of phenotypic flexibility? *Journal of Comparative Physiology B* 175, 453-461.

Seebacher, F., Guderley, H., Elsey, R.M., Trosclair, P.L., 2003. Seasonal acclimatisation of muscle metabolic enzymes in a reptile (*Alligator mississippiensis*). *Journal of Experimental Biology* 206, 1193-1200.

Seebacher, F., Holmes, S., Roosen, N. J., Nouvian, M., Wilson, R. S., & Ward, A. J. (2012). Capacity for thermal acclimation differs between populations and phylogenetic lineages within a species. *Functional Ecology*, 26(6), 1418-1428.

Seebacher, F., White, C. R., & Franklin, C. E. (2015). Physiological plasticity increases

resilience of ectothermic animals to climate change. *Nature Climate Change*, 5(1), 61-66.

Sibly, R. M. & Atkinson D. (1994). How rearing temperature affects optimal adult body size in ectotherms. *Functional Ecology* 8:486–493.

Sinervo, B., Mendez-De-La-Cruz, F., Miles, D. B., Heulin, B., Bastiaans, E., Villagrán-Santa Cruz, M., Lara-Resendiz R., Martinez-Mendez N., Calderon-Espinosa M. L., Meza-Lazaro, R. N., Gadsden, H., Avila, L., Morando, M., De La Riva, I., Sepulveda, P., Rocha, C., Ibarguengoytia, N., Puntriano, C., Massot, M., Lepetz, V, et al. & Sites, J. (2010). Erosion of lizard diversity by climate change and altered thermal niches. *Science*, 328(5980), 894-899

Sodhi, N. S., Brook, B. W., & Bradshaw, C. J. (2009). Causes and consequences of species extinctions. *The Princeton guide to ecology*, 1(1), 514-520.

Stapley, J., Reger, J., Feulner, P. G., Smadja, C., Galindo, J., Ekblom, R., Bennison, C., Ball, A. D., Beckerman, A. P. & Slate, J. (2010). Adaptation genomics: the next generation. *Trends in ecology & evolution*, 25(12), 705-712.

Sun, Y. B., Fu, T. T., Jin, J. Q., Murphy, R. W., Hillis, D. M., Zhang, Y. P., & Che, J. (2018). Species groups distributed across elevational gradients reveal convergent and continuous genetic adaptation to high elevations. *Proceedings of the National Academy of Sciences*, 115(45), E10634-E10641.

Supple, M. A., & Shapiro, B. (2018). Conservation of biodiversity in the genomics era. *Genome biology*, 19(1), 1-12.

Svensson, E. I., Gomez-Llano, M., & Waller, J. T. (2020). Selection on phenotypic plasticity favors thermal canalization. *Proceedings of the National Academy of Sciences*, 117(47), 29767-29774.

Thorpe, R. S., & Brown, R. P. (1989). Microgeographic variation in the colour pattern of the lizard *Gallotia galloti* within the island of Tenerife: distribution, pattern and hypothesis testing. *Biological Journal of the Linnean Society*, 38(4), 303-322.

Thorpe, R. S., & Brown, R. P. (1991). Microgeographic clines in the size of mature male *Gallotia galloti* (Squamata: Lacertidae) on Tenerife: causal hypotheses. *Herpetologica*, 28-37.

Thorpe, R. S., & Richard, M. (2001). Evidence that ultraviolet markings are associated with patterns of molecular gene flow. *Proceedings of the National Academy of Sciences*, 98(7), 3929-3934.

Vitales, D., Garnatje, T., Pellicer, J., Vallès, J., Santos-Guerra, A., & Sanmartín, I. (2014). The explosive radiation of *Cheirolophus* (Asteraceae, Cardueae) in Macaronesia. *BMC Evolutionary Biology*, 14(1), 1-15.

von Linné, C. (1744). *Oratio de Telluris habitabilis incremento*. Batavorum.

Wake, D. B., & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences*, 105(supplement_1), 11466-11473.

Wallberg, A., Schöning, C., Webster, M. T. and Hasselmann, M. (2017). Two extended haplotype blocks are associated with adaptation to high altitude habitats in East African honey bees. *PLOS Genetics* 13: e1006792.

Warren, B. H., Simberloff, D., Ricklefs, R. E., Aguilée, R., Condamine, F. L., Gravel, D., Morlon, H., Mouquet, N., Rosindell, J., Casquet, J., Conti, E., Cornuault, J., Fernández-Palacios, J. M., Hengl, T., Norder, S. J., Rijdsdijk, I. S., Strasberg, D., Triantis, K. A., Valente, L. M., Whittaker, R. J., Gillespie, R. G., Emerson, B. C., & Thébaud, C. (2015). Islands as model systems in ecology and evolution: Prospects fifty years after MacArthur-Wilson. *Ecology Letters*, 18(2), 200-217.

Woods, H. A. (1999). Egg-mass size and cell size: Effects of temperature on oxygen distribution. *American Zoologist*, 39, 244–252.

Yang, W., Qi, Y., & Fu, J. (2016). Genetic signals of high-altitude adaptation in amphibians: a comparative transcriptome analysis. *BMC genetics*, 17(1), 1-10.

Yang, W., Qi, Y., Lu, B., Qiao, L., Wu, Y., & Fu, J. (2017). Gene expression variations in high-altitude adaptation: a case study of the Asiatic toad (*Bufo gargarizans*). *BMC genetics*, 18(1), 1-8.

Žagar, A., Carretero, M. A., Marguč, D., Simčič, T., & Vrezec, A. (2018). A metabolic syndrome in terrestrial ectotherms with different elevational and distribution patterns. *Ecography*, 41(10), 1728-1739.

Žagar, A., Simčič, T., Carretero, M. A., & Vrezec, A. (2015). The role of metabolism in understanding the altitudinal segregation pattern of two potentially interacting lizards. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 179, 1-6.

Zamocky, M., Furtmüller, P.G., Obinger, C. (2008). Evolution of catalases from bacteria to humans. *Antioxid. Redox Signal.* 10, 1527–1548.

Zhang, Q., Gou, W., Wang, X., Zhang, Y., Ma, J., Zhang, H., Zhang, Y & Zhang, H. (2016). Genome resequencing identifies unique adaptations of Tibetan chickens to hypoxia and high-dose ultraviolet radiation in high-altitude environments. *Genome biology and evolution*, 8(3), 765-776.

Zhang, X., Witt, K. E., Bañuelos, M. M., Ko, A., Yuan, K., Xu, S., Nielsen, R., & Huerta-Sanchez, E. (2021). The history and evolution of the Denisovan-EPAS1 haplotype in Tibetans. *Proceedings of the National Academy of Sciences*, i(22), e2020803118.

Chapter II – Plastic functional responses in a lizard living along a steep altitudinal gradient

Plastic functional responses in a lizard living along a steep altitudinal gradient

Nina Serén^{*1,2,3}, Rodrigo Megía-Palma^{1,4}, Tatjana Simčič⁵, Fabio Guarino⁶, Catarina Pinho^{1,2}, Anamarija Žagar^{1,5}, Miguel A. Carretero^{1,2,3}

¹*CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal*

²*BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal*

³*Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, 4099-002 Porto, Portugal*

⁴*Consejo Superior de Investigaciones Científicas (CSIC) Spain*

⁵*National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia*

⁶*Dipartimento di Biologia, Università degli Studi di Napoli Federico II, Via Cinthia, 80126, Napoli, Italy*

2.1. Abstract

Physiological and metabolic performance are key mediators of a species' functional response to environmental variation. Few environments offer such a multifaceted array of stressors as high-altitude habitats, which relative to low-altitude habitats differ markedly in temperature, water availability, UV radiation and oxygen pressure. These patterns make species that inhabit altitudinal gradients excellent models to study how species respond to environmental variation. In this study we collected data on age structure, thermal and hydric regulatory behaviour and four metabolic and stress-related biomarkers for an insular lizard (*Gallotia galloti*) that inhabits an extreme altitudinal range (near sea-level to 3,700m), with the aim of understanding how survivability, ecophysiology and metabolism respond to extreme altitude in an ectotherm. Overall, we found marked differences in metabolic stress markers associated with altitude (particularly in the abundance of carbonyl metabolites, and relative telomere length), but with no 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 an altitudinal gradient. In lizard survivability or thermal physiological performance, we also did not find signs of an effect of altitude. Namely, across its altitudinal distribution, lizard populations have a similar age structure and thermal preferences. Mixed signals in life-history traits and thermal ecophysiological performance across populations and altitude suggest that response to variable conditions across altitude in this ectotherm could be associated with plasticity or local adaptation (meeting the genomic evidence) but further studies are needed to get a better grasp on the functional biology of adaptation to altitude in these ectotherms.

Key-words:

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

2.2. Introduction

The impact of climate and environmental change on biodiversity is a major scientific concern (Huey et al., 2012). Although this is a hot topic given the rapid pace of biodiversity loss (Ceballos et al., 2017), 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 advances to identify signals of these responses in several species are appearing during last years, which can be of importance for planning realistic conservation strategies (Mawdsley et al., 2009; Harmon & Pfennig, 2021).

An important strategy for understanding biological responses to environmental pressures can be targeting species that inhabit steep biological gradients. Altitudinal spatial gradients, in particular, can act as a proxy for recent 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, ultra-violet radiation), often within short geographic ranges, requires a suite of behavioural and physiological responses such as optimization of respiration, thermoregulation, physiology or changes in gut microbiota to account for differences in food types (Howald & Hoppeler, 2003; Solano et al., 2016; Žagar et al., 2018a; Quagliariello et al., 2019; Storz & Scott, 2019; González-Morales et al., 2021; Žagar et al., 2022).

In this context, ectothermic vertebrates are key models in environmental change studies. These animals have a strong dependency on their surrounding environment to regulate their body temperature (Angilletta, 2009; Huey, 1982; Sinervo et al., 2010) which should make them more vulnerable to environmental change (specifically climate) than endotherms (Paaijmans et al., 2013). Inversely, several studies pinpoint that ectothermic vertebrates appear to be fairly resilient to environmental change, especially if they have a great plastic ability to evolve (which is often the case, see Seebacher et al., 2015 and Crowell et al., 2021). Lizards in particular are useful for this type of research since they are sedentary and have short generation times making them easy targets to environmental change (Pianka & Vitt, 2003). Moreover, many are suitable for experimental manipulation, since they are easily captured and cope well with captivity in laboratory facilities (Pianka & Vitt, 2003).

Despite lizards inhabit places in a wide range of thermal conditions, a recent study from Garcia-Porta et al. (2019) with over 200 species of lacertids has shown an

existing correlation between physiology and climate, especially related with hydoregulation while thermal physiology remains phylogenetically conservative. This may reinforce earlier suggestions that lizards, particularly in temperate areas, may be susceptible to ongoing global warming (but see Muñoz 2021; Camacho et al., 2021). Environmental stress, in particular associated with climatic and altitudinal shifts, typically requires lizards to change their thermal and hydric regulatory responses to maintain fitness and performance (Cowles & Bogert, 1944; Angilletta et al., 2002; Angilletta, 2009). For example, studies show how dehydration in lizards is linked to reduced reproductive output and developmental rates (Lorenzon et al., 1999; Wang et al., 2016), requiring responses such as lowering the preferred body temperature or abandoning activity to prevent water loss (Sannolo & Carretero, 2019). Along altitudinal gradients information is still scarce, with a single study demonstrating that lizard populations at higher altitudes are more resistant to water loss but high parasite loads at lower elevations can make generalisation problematic (Sannolo et al., 2020).

Other abiotic factors apart from temperature (humidity, oxygen pressure, UV exposure) are also likely to influence metabolism of ectotherms since they impact on physicochemical processes (Cardinale et al., 2002; Rozen-Rechels et al., 2019; Rubalcaba et al., 2020). For lizards, the speed of metabolic response will depend on whether they show niche conservatism or plastic physiology (Schulte et al., 2012; Ahmadzadeh et al., 2013; Gangloff et al., 2019; Megía-Palma et al., 2020). Studies on metabolism and oxidative-stress markers can shed important light on the physiological responses of lizards to altitude (e.g. Seebacher et al., 2012; Žagar et al., 2015; Žagar et al., 2018a). For example, hypoxic environments at very high altitudes (> 3,000 m a.s.l.) are known to generate 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 lizards, it is still unclear if this is reflected in lower survivability, which is a complex question due to many other confounding pressures (such as predation pressure etc.: e.g. Martín & López, 1996).

Regardless of an animal's thermal regime, theory suggests that higher altitudes lead to a shift to a slower life-history strategy (reviewed in Laiolo & Obeso, 2017) which are often attributed to longer developmental periods, higher body mass and longer lifespan (Western & Ssemakula, 1982; Angilletta et al., 2004; Boyle et al., 2016; Masó et al., 2019; Roitberg et al., 2020). Environmental factors seem to be the main drivers to this differentiation, namely thermal amplitude, low temperatures, shorter activity

seasons, lower food availability, climate predictability and lower predation risk (Sandercock et al., 2005; Turbill et al., 2011; Laiolo & Obeso, 2017, 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 in squamates have found unpredicted shorter lifespan in medium altitudes comparing with the extremes of the studied gradient (Comas et al., 2020; Altunışık et al., 2022). Many of these contrasting results highlight how much still needs to be done to understand how species adapt to these extreme environments.

An additional widely used biomarker of oxidative stress is the telomere length of somatic cells, which not only is affected by stressors such as UV, immune challenges and thermal stress (Epel et al., 2004; Rochette & Brash, 2010; Debes et al., 2016; Evans et al., 2021), but also by age (Whittemore et al., 2019). Specifically in lizards, studies 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 positive (Fitzpatrick et al., 2021).

In this study we focused on the life history and ecophysiological responses to altitude in an endemic insular lizard species, *Gallotia galloti* (Oudart, 1839); (Figure 2.1a). This species is endemic to the islands of La Palma and Tenerife, in the Macaronesian archipelago of the Canary Islands. In Tenerife, the species inhabits a wide range of altitudes, from sea-level up to the summit of the Teide volcano (highest elevation of 3,718m a.s.l), experiencing this extreme gradient within approximately 30 km (Figure 2.1b, 2.1c). This island setting provides some advantages for studying environmental adaptation because it both reduces some of the complexity of environmental conditions and because island species usually experience less interspecific competition (Žagar et al., 2015; Jiménez-Robles & de la Riva, 2019). Currently, four species of reptiles are present in this island (Pleguezuelos et al., 2002), none of which thought to be either a potential competitor to *G. galloti* or sympatric at mid and high altitudes. A phylogeographic structuring between the North and South *G. galloti* populations is well documented since clear phenotypic and genetic differences are described (Bischoff, 1982; Brown et al., 2006, 2016, see also Chapter IV of this thesis) and this is why we focused our sampling to one slope of the island – the South. To assess how *G. galloti* responds along an extreme environmental gradient on the South of the island we collected data on the age, thermal and hydric physiological traits, and biomarkers of metabolic activity and stress of *G. galloti* from different altitudes. We hypothesize that

physiological stress associated with restrictive environmental conditions along the extreme altitudinal gradient may impose very different responses in life-history traits of *G. galloti*. Specifically, we predict that populations from the harsher high-altitude environments will: 1) experience changes in life-history strategy leading to shifts in age structure or signatures of reduced activity (Laiolo & Obeso, 2017); 2) select lower temperatures (Meiri et al., 2013, but see Sannolo et al., 2020) while water loss will correlate with humidity conditions across the altitude (highest humidity at mid-elevations); 3) have a higher antioxidant enzyme activity due to higher reactive oxygen species production under high UV (Žagar et al., 2018a) and 4) have a lower telomere size at higher altitudes due to stress (Bateson, 2016).

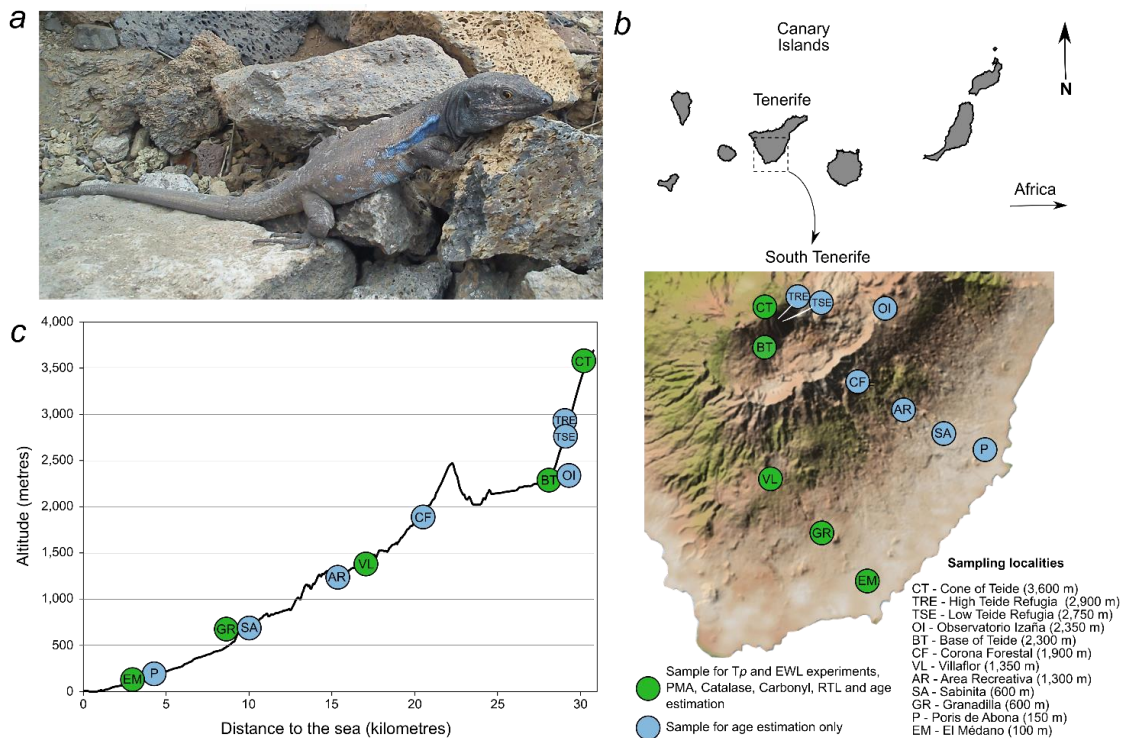


Figure 2.1. Altitudinal sampling design to study ecophysiology, metabolism, relative telomere length and age estimation through skeletochronology in an insular lizard. (a) The study species, *Gallotia galloti*, adult male. Photo by Nina Serén. (b) Geographical location of the island of Tenerife in the Canary Islands archipelago and a close-up of island topography showing the sampling sites. (c) Location of sampling sites along a topographic profile of the island of Tenerife from the sea to the highest point of the island (following the southeast sampling direction of the sampling).

2.3. Methods

Sampling

Samples of *G. galloti* in Tenerife were collected in two field missions held in July-August 2017 and 2018, synchronizing with the end of their reproductive season (Salvador, 2015). Both sampling periods were confirmed to be climatically very similar according to the climate data of Tenerife (<https://weatherspark.com>). The sampling strategy implemented in 2017 was an altitudinal transect where samples were collected for age estimation, ecophysiological experiments, metabolic activity and relative telomere length. The 2018 sampling was comprised of replicated and additional altitudinal locations where biological material was only collected for age estimation. Sampling was performed at an average of 630-metre intervals from the sea-level up to an altitude of approximately 3,550 m a.s.l. (Figure 2.1c). Sampling was restricted to adult males to avoid the reproductive cycle effects on ecophysiology, which are more intense and asynchronous in females due to vitellogenesis and pregnancy (e.g. Carretero, 2006), and collected between seven and 19 lizards per location (Table 2.1).

Table 2.1. Number of individuals from each sampling location in relation to the analysed variables. Abbreviations: T_p: preferred temperature; EWL: evaporative water loss; PMA: potential metabolic activity; RTL: relative telomere length.

Altitude (m a.s.l.)	Location	SVL	Weight	Age	T _p	EWL	PMA	Catalase	Carbonyl	RTL
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	-	-	-	-	-	-
1,300	AR	15	15	12	-	-	-	-	-	-
1,350	VL	16	16	12	17	17	17	17	17	17
1,900	CF	7	-	8	-	-	-	-	-	-
2,300	BT	15	15	9	15	15	13	15	13	15
2,350	OI	20	20	14	-	-	-	-	-	-
2,750	TSE	-	-	8	-	-	-	-	-	-
2,900	TRE	-	-	9	-	-	-	-	-	-
3,550	CT	12	9	11	7	7	7	7	7	13
	TOTAL	149	137	137	75	75	69	71	69	79

Lizards were collected by noose (García-Muñoz & Sillero, 2010) or using tomato and banana as bait in pitfall traps. Traps were animal-emptied approximately every 20 minutes to avoid undesired stress (Megía-Palma et al., 2016). Elevation and the

coordinates for each sampling site were registered with a GPS device (GPSMAP 64s, Garmin®, Kansas, USA). Adult males were carried to local laboratory facilities (Instituto de Productos Naturales y Agrobiología, IPNA, CSIC, altitude 560 m a.s.l.) to acclimatize in individual gradient terraria with water and food *ad libitum* for 12h. For each individual the snout–vent length (SVL) was recorded to the nearest 0.01 mm with digital callipers and the initial body weight (W_0) to the nearest 0.0001 g with a digital scale immediately before experimental ecophysiological procedures. After ecophysiological testing the 4th finger of one anterior limb for skeletochronology (with likely negligible effect on survival, Perry et al., 2011) and tail tip tissue for metabolic analyses were collected (these procedures are likely to have negligible effects on survival, see Perry et al., 2011 and Fernández-Rodríguez et al, 2021). Moreover, <50µL of blood were extracted from the caudal tail vein with sterile needles (BD microlance 3; 23G; 0.6 × 25 mm) from a previously ethanol-disinfected skin area and more than 2 cm away from the cloaca to avoid hemipenises. Fingers were stored in 96% ethanol; tail tips were immediately snap frozen in liquid nitrogen and blood was stored in 96% ethanol tubes and stored at 4° C. All lizards were released in the same location of trapping.

Skeletochronological analysis

To assess the age of the sampled males we applied a phalangeal skeletochronology methodology (Comas et al., 2016). After skin and surrounding muscle was carefully removed from the phalanges with the help of a binocular lens and ophthalmic scissors, phalanges were washed in running water and decalcified for approximately 90 minutes in a 5% nitric acid solution following standard protocols (Guarino et al. 1998). The decalcified phalanges were then prepared through a series of graded ethanol baths (three times 60 min absolute ethanol, then cleared with xylol, and finally embedded with paraffin with a 58 °C melting point). After the blocks of paraffin solidified, 10-12 mm thick cross sections were obtained with a manual-mechanic Leica 2035 microtome, and selected sections were stained with Harris hematoxylin with deparaffinization through an ethanol chain (5min xylene substitute, Neo-Clear®, Sigma-Aldrich®; 4 min xylene substitute and absolute ethanol 50:50; 1 min absolute ethanol; 1 min 96% ethanol, 2 min 75% ethanol; 5 min tap water, 15 min 1:4 Harris's hematoxylin). For each phalanx, at least four good quality, mid-diaphyseal sections (small medullar cavity and thick cortical bone) were selected under the microscope for careful observation of well-defined hematoxynophile lines (considered lines of arrested growth - LAGs) in the periosteal layer.

The number of LAGs, which correspond to the number of years, 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, if available, the second phalanx for the same individual was used. Since we had phalanx sections of nearly hatched *G. gallotia* provided by the museum collection of Koenig, we could apply the back-calculation method (Castanet et al. 1993; Guarino et al. 2008) to scale the LAGs and remove the uncertainty of endosteal resorption. A high variation in LAGs deposition was encountered in *G. galloti*, with double lines and false lines as documented by Castanet & Baez (1988, 1991), although this did not prevent age estimation in the majority of specimens. Despite encountered variation in arrested growth, sexual maturity was interpreted as a decrease of LAG spacing in many individuals at around 3-5 LAG-years, with a minor and less variable LAG spacing in the remaining countable sections. This assumption follows a similar interpretation as earlier skeletochronological studies on ectotherms (Leclair et al., 2000).

Thermoregulation experiments

Experiments to determine preferred body temperature (T_p) were performed with linear thermal gradients following the thermal gradient 10 h method in Koziel et al. (2021). Terraria with thermal gradients were built with PVC opaque walls with 100x30x40 cm and sandy soil was used as substrate (~ 1cm deep) with no shelter, food or water provided. This way each lizard could freely select the most adequate temperature throughout the experimentation time without thermoregulatory constraints (Veríssimo & Carretero, 2009). 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 to 68 °C. Lizards were kept under normal light photoperiod, with no direct sunlight but with natural light in a room at around 24° C (Koziel et al., 2021). Each lizard was randomly assigned to an individual terrarium and the infra-red-light bulb was preheated around 1h before each testing. Trials of lizards from different localities were also randomized. Cloacal temperature measurements were performed every hour for ten 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. The procedure did

not exceed 10 seconds to minimize stress and ensure more accurate temperature readings (Veríssimo & Carretero 2009).

Evaporative water loss experiments

Experiments of evaporative water loss (EWL) were performed the day after the preferred body temperature assays. In the intervening night, lizards were provided with a humid cloth to hydrate. 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 (48x38x30 cm) under constant humidity conditions (~25%) maintained by approximately 150 g of silica gel placed in a fabric net glued under the chamber lid (following the protocol of Ferreira et al., 2016). Temperature and humidity were measured with a Fluke®-971 hygrometer to the nearest 0.1 °C and 0.1%, respectively, to not vary far from 24 °C or 10-25% for standardization of the setting. These measurements were made every two hours for the species daily activity period (12 hours, six measurements from 7:00 to 17:00, WEST time zone). Each individual box without the false bottom was weighted using a digital balance (precision 0.0001 g) with measurements taken hourly for 12 consecutive hours (7:00-18:00).

Instantaneous water loss (EWL_i) values were obtained from an initial weight measurement recorded before the start of the experiment (W₀) and hourly records of weight (W_n) using the formula: $((W_n - W_{n+1}) / W_n) \times 100$. We also calculated the accumulated water loss (EWL_a) using the formula: $EWL_a = [W_0 - W_n] / W_0$. For subsequent analyses, we examined both the instantaneous water loss (EWL_i) relative to initial weight, W₀; using the formula $EWL_i = [(W_n - W_{n+1}) / W_0] \times 100$; and EWL_a in each time interval for each repeated trial measure. EWL_i captures the amount of EWL in each moment whereas EWL_a provides a measurement of the water lost by lizards throughout the experiment.

Metabolic activity assays

To test for potential metabolic activity (PMA) approximately 10 mm length from snap frozen tail tip tissue were collected to be homogenized with the help of a mortar and liquid nitrogen. The moist homogenized tissue was then weighted, and approximately 25 mg of wet weight was sonicated in 3 mL of ice-cold homogenization buffer 0.1 M sodium phosphate buffer pH = 8.4; 75 µM MgSO₄; 0.15% (w/v) polyvinyl

pyrrolidone; 0.2% (v/v) Triton-X-100) for 20 s (Cole-Parmer® ultrasonic homogenizer 4710) followed by a 4 min centrifugation at 0 °C and 10,000 rotations per minute (Centrifuge SIGMA® 2K15). Two replicates of 30 µL homogenate of the same sample and one blank without the homogenate were incubated simultaneously in plates filled with 150 µL of 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 of 2.5 mM 2-(p-iodophenyl)-3-(pnitrophenyl)-5- phenyl tetrazolium chloride (INT) solution). The incubation was carried out in parallel at three different incubator chambers during 20 minutes with three different temperature regimes (29 °C, 33 °C and 37 °C) around the median preferred body temperature, which in *G. galloti* is $T_p = 33.84$ °C, (see Supplementary Table S2.1). The reaction was terminated with 50 µL of stopping solution (formalin: H_3PO_4 concentration = 1:1 v/v). The final step to verify formazan production of the enzymatic process was determined spectrophotometrically from the absorbance of the sample at 490 nm against the control (microplate reader BioTek Instruments SynergyMX). From the spectrophotometry values, standard calculations were performed following Kenner and Ahmed (1975) to obtain a coarse estimate of PMA. The values of PMA were estimated as the INT reduction capacity ($\mu L O_2 mg^{-1} protein h^{-1}$) following the formula: $INT \text{ reduction capacity} = (ABS_{490} \times Vr \times Vh \times 60 \times 1.30) / (Va \times S \times t \times 1.42)$; where ABS_{490} is the absorption spectrum of the sample at a wavelength of 490 nm; 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 O_2 (Kenner & Ahmed, 1975).

Catalase activity was assessed following the method of Aebi (1984). The procedure started by mixing first 0.3 mL of the previously obtained tissue homogenate with 1.7 mL of 50 mM potassium phosphate buffer pH 7.0 and second adding to the mix 1 mL of 30 mM hydrogen peroxide. The latter was prepared in 50 mM potassium phosphate buffer pH 7.0, to give a concentration of hydrogen peroxide of 10 mM. Spectral analysis of the decomposition of the hydrogen peroxide was done by measuring absorbance at every 30 s at a wavelength of 240 nm for 3 min at 25 °C using quartz cuvettes with a path length of 10 mm and a Lambda UV/Vis spectrophotometer (PerkinElmer, Waltham, MA, USA). Enzyme activities were expressed in enzyme units (U), in which one U represents the amount of catalase that degrades 1 µmol of hydrogen peroxide in 1 min ($\epsilon_{240} = 43.6 M^{-1} cm^{-1}$). The obtained values were divided by the total amount of protein to give the specific catalase activity per mg protein. Total protein concentration of the homogenate was assessed with a Pierce™ BCA Protein Assay Kit

(Thermo Scientific, USA) using a microplate reader following the manufacturer's instructions.

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 375 nm by derivatising protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of stable dinitrophenyl (DNP)-hydrazone adducts proportional to the carbonyls present. Samples were homogenized in 500 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 used for analysis according to the manufacturer's instructions.

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. (2007), analysis was restricted to blood samples collected within a short temporal period (within 15 days) and stored in 96% ethanol at 4 °C. DNA extraction from blood was performed using the DNAextractme column-kit (BLIRT) following the manufacturer's instructions except for the overnight lysis (performed at 37 °C) and the final DNA elution volume (50 µL). Approximate DNA concentrations and quality were measured using a Nanodrop 8000 spectrophotometer (Thermo Scientific) and DNA integrity was assessed visually through agarose gel electrophoresis (1%). Concentrations were normalized before qPCR.

The qPCR amplification reactions were done using the SYBRGREEN (BioRad Labs) kit using the extracted DNA (15 ng), in 10 µl reactions and with telomere/reference primers at concentrations of 10 nM per primer. The selected primers for telomere amplification were Tel1 (5'-CGGTTTGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2 (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') from Callicott & Womack (2006), which use a modified version from the original designed primers by Cawthon (2002). As a reference 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'-CTCGATCCCAAGATCCAACT-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. The touchdown PCR profile for the amplification of the 18S fragment and telomeric region was the same: 95 °C for 3 min; 20 cycles of 95 °C for 30 sec, 64 °C for 30 sec, and 72 °C for 30 sec with a touchdown of the annealing temperature until 59 °C; 15 cycles of 92 °C for 30 sec, 59 °C for 30 sec and 72°C for 30 sec; and a final step of 72°C for 10 min. Three qPCR replicates were performed for each sample, and quantitation cycle values (Cq) 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. The calibrator sample (randomly selected) was serially diluted (from 0.02 to 50 ng) to produce a standard curve for each plate. 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 Supplementary Table S2.2, Figure S2.1). 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.

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 $P < 0.05$. The analyses to verify basic morphometric relations were performed using a one-way analysis of variance (1-way ANOVA) (*aov* function in *R*) using SVL and Weight separately as a proxy of size as a dependent variable and location as an independent variable. All standardized measures were transformed using log10 transformations. Robustness (weight relative to length) across different locations was assessed with ANCOVA using weight as dependent variable and SVL as covariate.

After these analyses, allometric effects of body size on all numerical values of biochemical and physiological variables were removed using Thorpe-Lleonart's method (Lleonart 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 identified as extreme outliers for each dataset using Tukey's method (if they were larger than the third quartile + $3 \times$ Interquartile range or smaller than the 1st quartile - $3 \times$ Interquartile range) (*identify_outliers* function in *rstatix* v.0.7.0 package in *R*) were removed from statistical analyses. Final sample sizes for analysed variables per location are discriminated in Supplementary Table S2.3. 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 from homoscedasticity). Assumption of sphericity for variables with repeated measures was checked with Mauchley's tests (no significant deviations in any variable).

The main objective of these analyses was to check for significant differences in age, physiological and biochemical 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: 1-way ANOVA (*aov* function in *R*) for age or Welch's ANOVA for non-homoscedastic variables (*oneway.test* function in *R*), 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) a two-way repeated measures ANOVA (2-way RM-ANOVA, *anova_test* function in *rstatix* v.0.7.0 package in *R*) strategy was implemented using location 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-Hockberg procedure to reduce the false discovery rate at $P < 0.05$. To test for the existence of linear or nonlinear relationships between single variables we used 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 interval (CI) of the α 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 ordinary least squares regressions).

2.4. Results

Body size comparison

Populations differed significantly in morphological traits, since SVL, weight and robustness (weight accounting for SVL) were all significant ($P < 0.001$; Table 2.2). However, significant differences between populations did not show any linear trend with altitude (Supplementary Tables S2.4, S2.5; Supplementary Figures S2.2, S2.3) although pairwise differences show that the lowest sampled population (EM) has significantly lower weight than all other, except one at mid altitude (VL), while SVL was the lowest in mid-altitude population (CF).

Similar age structure along the altitudinal cline

Age estimation through counting of LAGs in histological cuts of phalanx (Figure 2.2a) was performed on 119 lizards; histological prepared sections from an additional 18 lizards were discarded due to bad quality sections that made LAG counting impossible (total sample size was 138). This enabled to inspect whether populations inhabiting different altitudes show differences in age structure. 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. Skeletochronological age estimation confirmed that only 1.7% ($n=2$) of all presumed adults collected for this study (by absence of striped colouration typical from youngsters) were not yet sexually mature (ages 2 and 3 years) and these samples were removed from all subsequent analyses. 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 2.2b, Supplementary Table S2.6). Age structure showed some apparent variation between populations, but with no significant differences (Figure 2.2c, Table 2.2).

Table 2.2. 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*. Abbreviations: SVL – snout-vent length; T_p - preferred temperature; EWL_i - instantaneous evaporative water loss; EWL_a - accumulated evaporative water loss; PMA - potential metabolic activity; RTL - relative telomere length; LOC: sampling location. Showing Generalized eta squares for Repeated Measures ANOVA and Sum squares for ANCOVA and 2-way ANOVA.

		Effect	Total df	DFn	DFd	F	Generalized eta squares/ Sum of squares	P value
1-way ANOVA	SVL	LOC	164	11	153	9.287	-	< 0.001
	Weight	LOC	128	8	120	6.185		< 0.001
	Age	LOC	116	10	106	1.069	-	0.394
ANCOVA	Robustness	WEIGHT	1			263.521	0.058	< 0.001
		LOC	5			3.275	0.004	< 0.001
		Residuals	77				0.017	
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
2-way Repeated Measures ANOVA	T_p	LOC		4	58	0.515	0.010	0.725
		HOURL		6.84	396.71	9.557	0.106	< 0.001
		LOC:HOURL		27.36	396.71	0.863	0.041	0.668
	EWL_i	LOC		4	60	1.466	0.048	0.224
		HOURL		5.78	347.04	7.569	0.057	< 0.001
		LOC:HOURL		23.14	347.04	1.303	0.040	0.161
	EWL_a	LOC		4	68	2.547	0.110	0.047
		HOURL		1.97	133.89	461.486	0.540	< 0.001
		LOC:HOURL		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

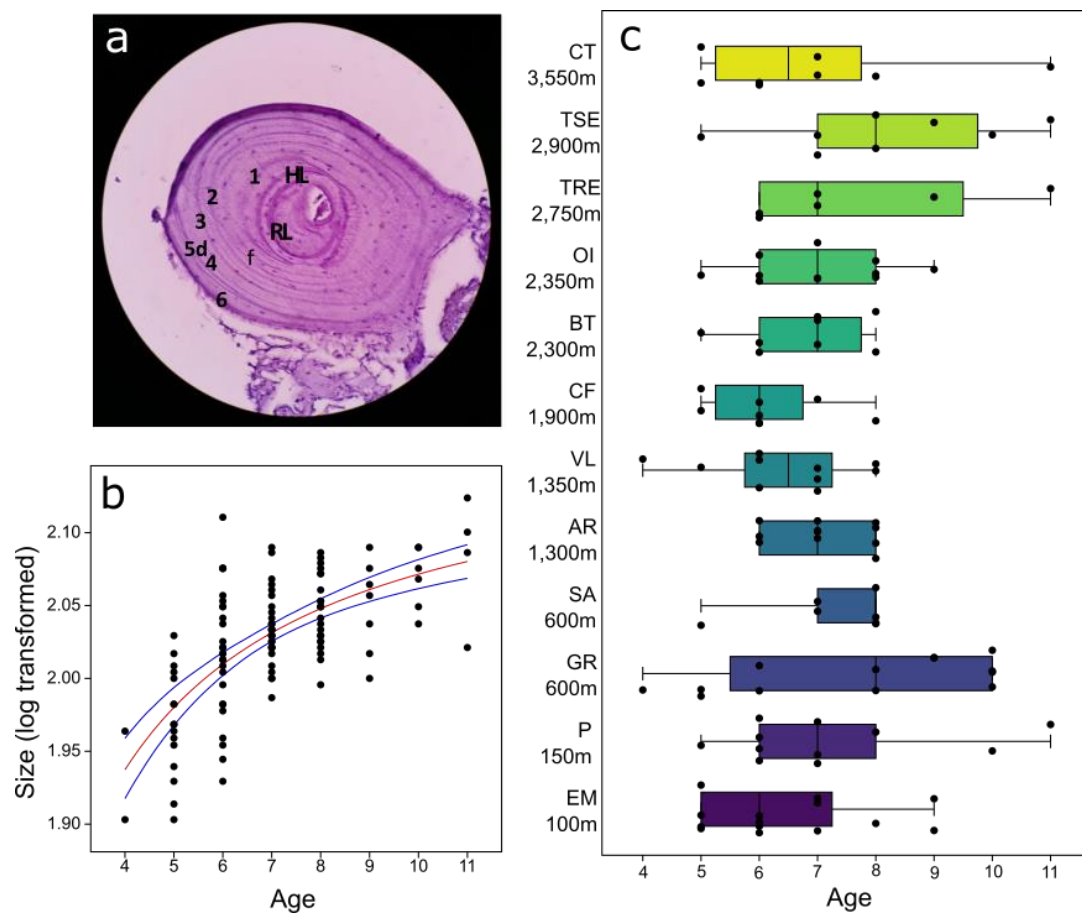


Figure 2.2. (a) Example of age estimation based on skeletochronology of growth rings in the phalanx of one analysed sample of *G. galloti*. Abbreviations: HL: hatching line; RL: reversal line; f: false LAG (line of arrested growth); 5d: 5th counted LAG-year, which is very close to the previous LAG and seems like a double line (top left). (b) Scatterplot comparing skeletochronological age estimation with (log) 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).

Thermo- and hydro-regulatory profiles across the altitude

The thermal preferences of *G. galloti* were significantly different between daily time-points (Table 2.2, Figure 2.3c); however, all tested populations along the altitudinal cline show the same thermoregulatory profile, both in terms of preferred temperatures and hourly patterns (Table 2.2 and Figure 2.3c). Hourly variation of preferred body temperature has a U-shape tendency in all populations, suggesting that *G. galloti* select for higher temperatures in the beginning and end of the day and slightly down-regulate their inner temperature at the hours of the day in which environmental temperature are higher.

Patterns of instantaneous evaporative water-loss varied significantly during the day (Table 2.2, Figure 2.3d) but with no significant differences between sampling locations. EWL_a was different between time-points (Figure 2.3e) but also differed significantly between locations ($F_{4,68}=2.547$, $P < 0.05$; Table 2.2). Pairwise comparisons (Supplementary Tables S2.7 and S2.8) indicate that the main between-location differences are not consistently related to altitude, but are associated with specific pairwise differences between measurements of a near high-altitude population (BT) against a mid-altitude population (VL) and a low-altitude population (EM).

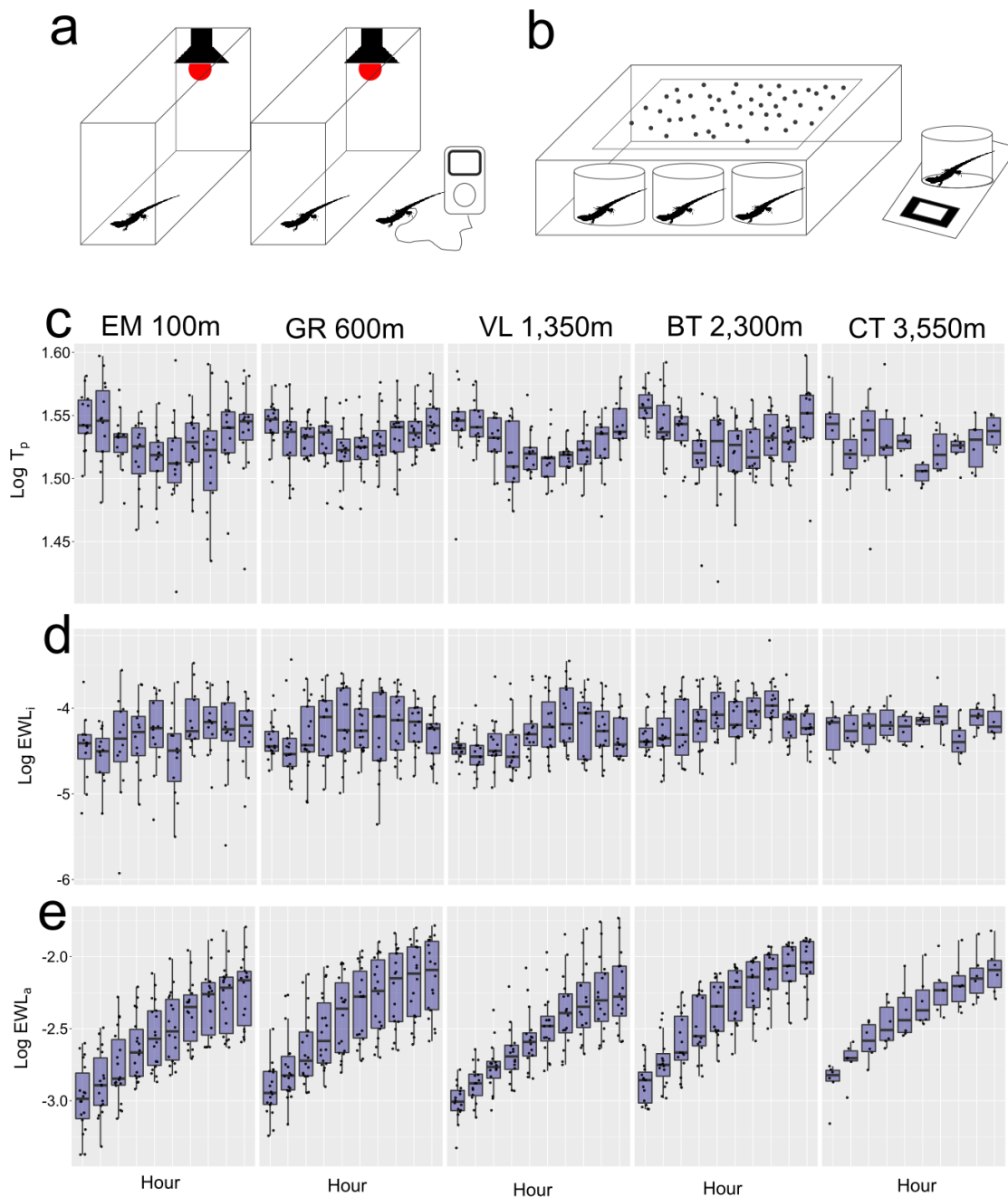


Figure 2.3. Experimental set ups for studying thermal preferences and evaporative water loss, and results of both experiments. (a) For experiments on thermal preferences lizards were placed in terraria heated with an infrared lightbulb at one end, allowing them to choose the distance to the heat source to thermoregulate; 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 weight due to water loss. (c) Location profiles of thermal preferences along the different periods of the day. (d) Location profiles of instantaneous water loss (EWL_i) along the different periods of the day. (e) Location profiles of accumulated water loss (EWL_a) along the different periods of the day. In each graph, populations are arranged (left-to-right) from the lowest altitude to the highest altitude. All variables are controlled for size and log-transformed.

Variable signals of biochemical traits of metabolism and stress at high altitude

To assess differences in stress-related biomarkers in populations inhabiting different altitudes we measured PMA, catalase activity, accumulation of carbonyl metabolites and RTL. PMA exhibited significant variation across test temperatures and locations (Table 2.2, Figure 2.4a). Pairwise comparisons show that the population GR (low-altitude population at ~600 m a.s.l.) is the main driver of this significant effect, as PMA is higher in this population compared to two sampled populations at the highest altitudes (BT at ~2300 m and CT at ~3550 m a.s.l., $P < 0.01$; Supplementary Table S2.9). Catalase activity levels did not vary between locations (Table 2.2, Figure 2.4b, Supplementary Table S2.10). Carbonyl protein content showed significant changes between populations ($P < 0.01$; Table 2.2, Figure 2.4c), driven by differences between the locations at lowest (EM, highest value) and highest (CT, lowest value) altitudes (Supplementary Table S2.11). RTL was significantly different between locations ($P < 0.01$; Table 2.2, Figure 2.4d): RTL was higher in the highest altitude population (CT), with significant differences to most other populations (except VL) (Supplementary Table S2.12). We 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 ($AIC_{\text{linear}} = 7.34$; OLS $P = 0.853$) or SVL ($AIC_{\text{Michaelis-Menten}} = 11.35$; $a = 0.02 \pm 3.02E06$ 95% CI) (Supplementary Table S2.13 and Figure S2.4).

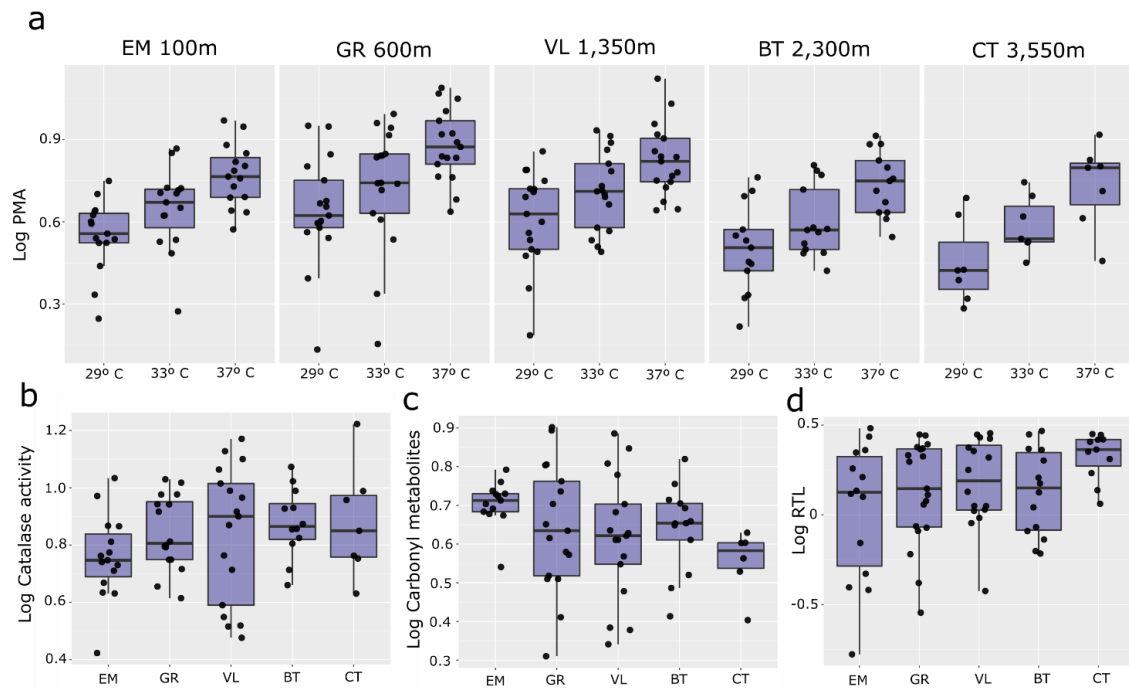


Figure 2.4. Box-and-jitter plots of studied physiological traits and relative telomere length (RTL). (a) Plots showing patterns of potential metabolic activity (PMA) in populations at different altitudes, with three distinct testing temperatures (29 °C, 33 °C and 37 °C). (b) Plots showing patterns of 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.

2.5. Discussion

Understanding how ectotherms respond and eventually adapt with increasing altitudes is of extreme relevance in evolutionary biology, not only to better understand how they thrive under conditions of increasing environmental harshness, but also to predict how endangered species can respond under rapid global environmental and climate change. In this study we show how, for a single lizard species inhabiting an extreme altitudinal gradient, some measures of functional performance display different patterns of variation along an increasing altitudinal cline and some even remain invariable. Moreover, differences between lower and higher altitude populations in stress-related biomarkers (carbonyl metabolites and telomere length) have been detected. Age structure is similar, further indicating that populations at higher altitude show similar life-history strategies as those from lower altitudes. Overall, our results suggest that colonization along this extreme altitudinal gradient for this insular lizard species could depend on phenotypic plasticity, since lizards placed under similar experimental conditions seem to select for similar physiological optima.

Phenotypic plasticity represents the fast-acting responses to environmental change compared to adaptation that requires time for both genetic variation (either through mutation or migration) and frequency to arise due to natural selection. Traditional views of plasticity place it as an opposite force to genetic adaptation, not only because phenotypic differences arise with minimal genetic contribution, but also because plasticity is many times thought of as potentially delaying evolution, buffering the effects of selection on genetic variants (Ghalambor et al., 2007). However, increasing evidence shows that plasticity may also promote evolution, because it creates an opportunity for genotypic variability and selection to “catch up” under ongoing change and promote longer term adaptation (Ghalambor et al., 2015; Levis & Pfennig, 2016; Corl et al., 2018). These complex relationships between selection and plasticity for organismal response to environmental change show why the importance of phenotypic plasticity for conservation is increasingly recognized in the context of fast and striking biodiversity loss (Fox et al., 2019). In *G. galloti* lizards sampled at locations at different altitudes, their preferred temperatures when placed in similar laboratorial conditions are similar. This result may indicate that these lizards may need to devote a different proportion of their activity to thermoregulation because they all seem to need to achieve similar 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. 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 some other lizard species (Jiang et al., 2021; Bodensteiner et al., 2021; but see for example Díaz de la Vega-Pérez et al., 2019 and Camacho et al., 2018 for a similar scenario to ours). Nevertheless, there might be other advantageous conditions contributing to expansion upwards, such as lower predation pressure near the volcano summit, that are not yet explored. Moreover, most likely the top populations do not act as a sink population because the age structure of those populations does not differ from lowlands. In any case, it should be noted that further tests should be conducted to support the plasticity hypothesis, in particular conducting translocation experiments to understand the capacity of individuals to adjust to changed environments.

Contradicting the absence of variation in thermal physiology, hydric physiology revealed differentiation between populations of the studied gradient. Changes in body size between populations may arise due to local variation in life-history and food availability, and subsequently can contribute to differences in water loss rates (smaller lizards have a higher surface-to-volume ratio and thus a higher rate of water loss across the skin). Changes in precipitation within the altitudinal microclimates, known for the island and that do not vary linearly with altitude (Juan et al., 2000) could also have caused the found variation between certain populations.

Previous studies have shown that in the range of preferred body temperatures of ectotherm organisms, potential metabolic activity increases with increasing body temperature (e.g., Simčič and Brancelj, 1997; Simčič et al., 2014; Bielen et al., 2016), 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). In our study, we detected differences in potential metabolic activity between populations across the elevation, 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 elevations may be related with the avoidance to the combined effects of stressing high elevation conditions (hypoxia and high UV). During aerobic respiration there is a default rate of by-production of reactive oxygen species (ROS, Halliwell, 1989), and because of

its damaging effects, antioxidant mechanisms are simultaneously activated to prevent oxidative stress (Monaghan et al., 2009). Previously it has been found in white-worms (*Enchytraeus albidus*) that low basal metabolism is selected for in freeze-tolerant organisms where winter survival is important for fitness as a mechanism of avoidance of the additive effect of ROS production from metabolism and freezing (Žagar et al., 2018b). This could also be the case in populations at the high altitudes in Tenerife, which undergoes regular winter snow, and where selection for low basal metabolism would act as an avoidance mechanism of the additive effect of ROS production from metabolism under strong UV and hypoxia conditions.

As verified in other studies with telomere length in ectothermic vertebrates, age and telomere length were uncorrelated (Lund et al., 2009; Gao & Munch, 2015). 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 studies arguing for negligible senescence in reptiles (da Silva et al., 2022; Reinke et al., 2022)). Ectotherm studies have shown that with ageing telomere length either decreases (Scott et al., 2006), increases (Axelsson et al., 2020) or shows a variable pattern as in our system (Lund et al., 2009; Gao & Much, 2015). 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 analyze the trends of telomere length across the altitude. We found a significant increase of relative telomere length with altitude and a significant inverse relation of telomere length with carbonyl metabolites (which are stress-indicator proteins). 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 highest altitude populations can cope with harsh UV and hypoxic conditions without reflecting stress by biochemical markers such as carbonyl. It is possible that genetic adaptations provide a means to cope with these factors, as suggested in our genomic analyses (see Chapter III).

Furthermore, for telomere lengths, recent articles have shown how a series of life-history changes seem to counter-balance the classic logic of 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 (Hille & Cooper, 2015; Giraudeau et al., 2019), 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) also found longer telomeres in high altitude 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 metabolic adaptation to these environments, but may also result from the life-history strategies mentioned above that prevent oxidative stress at high-altitude. On the other hand, higher carbonyl content detected in lowland populations could be due to higher stress due to dry conditions of the lowland *G. galloti* population. However, it should be stressed out that water availability does not vary predictably with altitude (in opposition to other environmental factors, such as oxygen pressure). Therefore, more work needs to be pursued 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). Since long-term hypoxia is more difficult to counteract through plastic changes in behaviour or physiology, this suggests that selection on genes involved in response to hypoxia can also have an important role important for adaptation to these extreme conditions. This is consistent with our findings on stronger signatures of selective sweeps near genes associated with response to hypoxia (see Chapter III of this thesis). Although further studies are needed to better interpret the functional biology of adaptation to altitude in these ectotherms, our results show that a complex landscape of genetic adaptation and plasticity explains how these animals cope with extreme conditions.

2.6. Acknowledgements

NS was funded by a PhD scholarship (SFRH/BD/128612/2017) and extension FCT grant (COVID/BD/152580/2022) attributed by Fundação para a Ciência e Tecnologia. CP was supported by Portuguese national funds through FCT-Fundação para a Ciência e a Tecnologia in the scope of the project UIDB/50027/2020. MAC was supported by the project NORTE-01-0145-FEDER-000007. This project is funded by a Portugal 2020 FCT project (2801402/SAICT/2017). AŽ and TS were funded from the state budget by the Slovenian Research Agency (Programme P1-0255 and project grant J1-2466). We are thankful to Manuel Nogales, current director of IPNA-CSIC (Spanish National Research Council - Department of Agrobiology and the Environment) for all the support and providing an ecology lab to conduct the experiments at La Laguna (Tenerife, Spain). Thank you to M. Lazić and D. Rödder from Museum Alexander Koenig (Bonn) for having provided juvenile toe samples from *G. gallotia*. A very special thanks for precious help in the field work carried out in Tenerife, to Julien Piquet, Miha Krofel, Nayra Hernández-Acosta, Senda Reguera and Xavier Santos.

2.7. References

- Aebi, H. (1984) Catalase in Vitro. *Methods in Enzymology*, 105, 121-126.
- Ahmadzadeh, F., Flecks, M., Carretero, M.A., Böhme, W., Ilgaz, C., Engler, J.O., Harris, D.J., Üzümlü, N., Rödder, D. (2013). Rapid lizard radiation lacking niche conservatism: ecological diversification within a complex landscape. *Journal of Biogeography* 40, 1807–1818.
- Albaladejo-Robles, G., Rodríguez, N., Rodríguez-Concepción, B., Nogales, M., & Vences, M. (2022). Limited ecophysiological variation in *Gallotia galloti* (Oudart, 1839) lizards across an elevational gradient of over 3,500 meters (Squamata: Lacertidae). *Herpetology Notes*, 15, 87-96.
- Altunışık, A., Yıldız, M. Z., Üçeş, F., Bozkurt, M. A., & Sömer, M. (2022). Altitude impacts body size but not age in the Asia Minor Thin-toed Gecko (*Mediodactylus heterocercus*). *Canadian Journal of Zoology*, 100(999), 315-321.
- Andrade, P., Pinho, C., i de Lanuza, G. P., Afonso, S., Brejcha, J., Rubin, C. J., Wallerman, O., Pereira, P., Sabatino, S. J., Bellati, A., Pellitteri-Rosa, D., Bosakova, Z., Bunikis, I., Carretero, M. A., Feiner, N., Marsik, P., Paupéio, F., Salvi, D., Soler, L., While, G. M., Uller, T., Font, E., Andersson & Carneiro, M. (2019). Regulatory changes in pterin and carotenoid genes underlie balanced color polymorphisms in the wall lizard. *Proceedings of the National Academy of Sciences*, 116(12), 5633-5642.
- Angilletta Jr, M. J., Steury, T. D., & Sears, M. W. (2004). Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. *Integrative and comparative biology*, 44(6), 498-509.
- Angilletta MJ, Niewiarowski PH, Navas CA. (2002) The evolution of thermal physiology in ectotherms. *Journal of thermal biology*. 2002; 27:249–268.
- Angilletta MJ. (2009). Thermal Adaptation: A Theoretical and Empirical Synthesis. Oxford University Press Inc New York, USA.
- Axelsson J, Wapstra E, Miller E, Rollings N, Olsson M. (2020). Contrasting seasonal patterns of telomere dynamics in response to environmental conditions in the ectothermic sand lizard, *Lacerta agilis*. *Scientific Reports* 10, 182.
- Båmstedt, U. (1980). ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. *Journal of*

Experimental Marine Biology and Ecology, 42: 267–283.

Bateson, M. (2016). Cumulative stress in research animals: Telomere attrition as a biomarker in a welfare context?. *BioEssays*, 38(2), 201-212.

Bielen, A., Bošnjak, I., Sepčić, K., Jaklič, M., Cvitanić, M., Lušić, J., Lajtner, J., Simčič, T., & Hudina, S. (2016). Differences in tolerance to anthropogenic stress between invasive and native bivalves. *Science of the Total Environment*, 543, 449-459.

Bischoff, W. (1982): Die innerartliche Gliederung von *Gallotia galloti* (Dumeril & Bibron, 1839) (Reptilia: Sauria: Lacertidae) auf Teneriffa, Kanarische Inseln. *Bonner zoologische Beiträge*, 33(2–4): 363–382.

Bodensteiner, B. L., Gangloff, E. J., Kouyoumdjian, L., Muñoz, M. M., & Aubret, F. (2021). Thermal–metabolic phenotypes of the lizard *Podarcis muralis* differ across elevation, but converge in high-elevation hypoxia. *Journal of Experimental Biology*, 224(24), jeb243660.

Boyle, W. A., Sandercock, B. K., & Martin, K. (2016). Patterns and drivers of intraspecific variation in avian life history along elevational gradients: a meta-analysis. *Biological Reviews*, 91(2), 469-482.

Brown, R. P., Hoskisson, P. A., Welton, J. H., & Baez, M. (2006). Geological history and within-island diversity: a debris avalanche and the Tenerife lizard *Gallotia galloti*. *Molecular Ecology*, 15(12), 3631-3640.

Brown, R. P., Paterson, S., & Risse, J. (2016). Genomic signatures of historical allopatry and ecological divergence in an island lizard. *Genome biology and evolution*, 8(11), 3618-3626.

Burraco, P., Comas, M., Reguera, S., Zamora-Camacho, F. J., & Moreno-Rueda, G. (2020). Telomere length mirrors age structure along a 2200-m altitudinal gradient in a Mediterranean lizard. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 247, 110741.

Cabezas-Cartes, F., Boretto, J. M., & Ibargüengoytia, N. R. (2018). Effects of climate and latitude on age at maturity and longevity of lizards studied by skeletochronology. *Integrative and Comparative Biology*, 58(6), 1086-1097.

Callicott, R. J., & Womack, J. E. (2006). Real-time PCR assay for measurement of mouse telomeres. *Comparative medicine*, 56(1), 17-22.

Camacho, A., Rodrigues, M. T., Jayyusi, R., Harun, M., Geraci, M., Vinagre, C.,

- Carretero, M. & Tejedo, M. (2021). Does heat tolerance actually predict animals' geographic thermal limits?. *bioRxiv*.
- Camacho, A., Vandenbrooks, J. M., Riley, J., Telemeco, R. S. and Angilletta, M. J. jr. (2018). Oxygen supply did not affect how lizards responded to thermal stress. *Integrative Zoology* 13: 428-436.
- Cardinale, B. J., Palmer, M. A., Swan, C. M., Brooks, S., & Poff, N. L. (2002). The influence of substrate heterogeneity on biofilm metabolism in a stream ecosystem. *Ecology*, 83(2), 412-422.
- Carretero, M. A. (2006): Reproductive cycles in Mediterranean lacertids: Plasticity and constraints. pp. 33-54, in: Corti, C.; Lo Cascio, P. & Biaggini M.(eds.) *Mainland and insular lacertid lizards: a Mediterranean perspective*. Firenze University Press.Florence.
- Castanet, J., & Baez, M. (1988). Data on age and longevity in *Gallotia galloti* (Sauria, Lacertidae) assessed by skeletochronology. *Herpetological Journal*, 1(06), 218-222.
- Castanet, J., & Baez, M. (1991). Adaptation and evolution in *Gallotia* lizards from the Canary Islands: age, growth, maturity and longevity. *Amphibia-Reptilia*, 12(1), 81-102.
- Castanet, J., Francillon-Vieillot, H., Meunier, F. J., de Ricqles, A. (1993). Bone and individual aging. In: Hall BK (Ed.) *Bone Growth* (Vol. 7). CRC, Boca Raton, 245–283.
- Cawthon, R. M. (2002). Telomere measurement by quantitative PCR. *Nucleic acids research*, 30(10), e47-e47.
- Ceballos, G., Ehrlich, P. R., & Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proceedings of the National Academy of Sciences*, 114(30), E6089-E6096.
- Comas, M., Reguera, S., Zamora-Camacho, F. J., & Moreno-Rueda, G. (2020). Age structure of a lizard along an elevational gradient reveals nonlinear lifespan patterns with altitude. *Current Zoology*, 66(4), 373-382.
- Comas, M., Reguera, S., Zamora-Camacho, F. J., Salvado, H. H., & Moreno-Rueda, G. (2016). Effectiveness of phalanx skeletochronology to estimate age in living reptiles (No. e1809v1). *PeerJ Preprints*.
- Corl, A., Bi, K., Luke, C., Challa, A. S., Stern, A. J., Sinervo, B., & Nielsen, R. (2018). The genetic basis of adaptation following plastic changes in coloration in a novel environment. *Current Biology*, 28(18), 2970-2977.

Cowles, R. B., & Bogert, C. M. (1944). A preliminary study of the thermal requirements of desert reptiles. *Bulletin of the AMNH*; v. 83, article 5.

Crowell, H. L., King, K. C., Whelan, J. M., Harmel, M. V., Garcia, G., Gonzales, S. G., Maier, P. H., NeldnerH., Nhu, T., Nolan, J. T., & Taylor, E. N. (2021). Thermal ecology and baseline energetic requirements of a large-bodied ectotherm suggest resilience to climate change. *Ecology and Evolution*, 11(12), 8170-8182.

da Silva, R., Conde, D. A., Baudisch, A. and Colchero, F. 2022. Slow and negligible senescence among testudines challenges evolutionary theories of senescence. - *Science* 376: 1466-1470.

Dagnall, C. L., Hicks, B., Teshome, K., Hutchinson, A. A., Gadalla, S. M., Khincha, P. P., Yeager, M., & Savage, S. A. (2017). Effect of pre-analytic variables on the reproducibility of qPCR relative telomere length measurement. *PloS one*, 12(9), e0184098.

Debes, P. V., Visse, M., Panda, B., Ilmonen, P., & Vasemägi, A. (2016). Is telomere length a molecular marker of past thermal stress in wild fish?. *Molecular Ecology*, 25(21), 5412-5424.

Díaz de la Vega-Pérez, A. H., Barrios-Montiel, R., Jiménez-Arcos, V. H., Bautista, A., & Bastiaans, E. (2019). High-mountain altitudinal gradient influences thermal ecology of the Mesquite Lizard (*Sceloporus grammicus*). *Canadian Journal of Zoology*, 97(8), 659-668.

Epel, E. S., Blackburn, E. H., Lin, J., Dhabhar, F. S., Adler, N. E., Morrow, J. D., & Cawthon, R. M. (2004). Accelerated telomere shortening in response to life stress. *Proceedings of the National Academy of Sciences*, 101(49), 17312-17315.

Evans, J. R., Torres-Pérez, J. V., Miletto Petrazzini, M. E., Riley, R., & Brennan, C. H. (2021). Stress reactivity elicits a tissue-specific reduction in telomere length in aging zebrafish (*Danio rerio*). *Scientific reports*, 11(1), 1-11.

Fernández-Rodríguez, I.; Barroso, F. M. & Carretero, M. A. (2021): An integrative analysis of the short-term effects of tail autotomy on thermoregulation and dehydration rates in wall lizards (*Podarcis bocagei*). *Journal of Thermal Biology*. 99: 102976.

Ferreira, C. C., Santos, X., & Carretero, M. A. (2016). Does ecophysiology mediate reptile responses to fire regimes? Evidence from Iberian lizards. *PeerJ*, 4, e2107.

Fitzpatrick, L. J., Olsson, M., Pauliny, A., While, G. M., & Wapstra, E. (2021). Individual telomere dynamics and their links to life history in a viviparous lizard. *Proceedings of the*

Royal Society B, 288(1951), 20210271.

Fox, R. J., Donelson, J. M., Schunter, C., Ravasi, T., & Gaitán-Espitia, J. D. (2019). Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. *Philosophical Transactions of the Royal Society B*, 374(1768), 20180174

Gangloff, E. J., Sorlin, M., Cordero, G. A., Souchet, J., & Aubret, F. (2019). Lizards at the peak: Physiological plasticity does not maintain performance in lizards transplanted to high altitude. *Journal of Physiology and Biochemistry Zool*, 92, 189-200.

Gao, J., & Munch, S. B. (2015). Does reproductive investment decrease telomere length in *Menidia menidia*? *PLoS ONE*, 10, e0125674.

García-Muñoz, E., & Sillero, N. 2010. Two new types of noose for capturing herps. - *Acta Herpetologica* 5: 259-263

Garcia-Porta, J., Irisarri, I., Kirchner, M., Rodríguez, A., Kirchhof, S., Brown, J. L., MacLeod, A., Turner, A. P., Ahmadzadeh, F., Albaladejo, G., Crnobrnja-Isailovic, J., De LA Riva, I., Fawzi, A., G., P., Göçmen, B., Harris, D. J., Jiménez-Robles, O., Joger, U., Jovanović Glavaš, O., Karış, M, et al. & Valero, K. C. W. (2019). Environmental temperatures shape thermal physiology as well as diversification and genome-wide substitution rates in lizards. *Nature communications*, 10(1), 1-12.

Gaur, P., Prasad, S., Kumar, B., Sharma, S. K., & Vats, P. (2021). High-altitude hypoxia induced reactive oxygen species generation, signaling, and mitigation approaches. *International Journal of Biometeorology*, 65(4), 601-615.

Ghalambor, C. K., Hoke, K. L., Ruell, E. W., Fischer, E. K., Reznick, D. N., & Hughes, K. A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature*, 525(7569), 372-375.

Ghalambor, C. K., McKay, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, 21(3), 394-407.

Giraudeau, M., Angelier, F., & Sepp, T. (2019). Do telomeres influence pace-of-life-strategies in response to environmental conditions over a lifetime and between generations?. *BioEssays*, 41(3), 1800162.

González-Morales, J. C., Rivera-Rea, J., Moreno-Rueda, G., Bastiaans, E., Díaz-Albiter, H., Díaz de la Vega-Pérez, A. H., Bautista, A., & Fajardo, V. (2021). To be small and dark is advantageous for gaining heat in mezquite lizards, *Sceloporus grammicus*

(Squamata: Phrynosomatidae). *Biological Journal of the Linnean Society*, 132(1), 93-103.

Guarino F. M., Andreone F., Angelini F., 1998 - Growth and longevity in *Mantidactylus microtympaanus*, a rainforest anuran from southern Madagascar. *Copeia*, 1998: 194-198.

Guarino FM, Di Già I, Sindaco R (2008) Age structure in a declining population of *Rana temporaria* from northern Italy. *Acta Zoologica Academiae Scientiarum Hungaricae* 54: 99–112.

Halliwell, B. (1989). Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *British journal of experimental pathology*, 70(6), 737.

Hammer, Ø., Harper, D. A., & Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia electronica*, 4(1), 9.

Harmon, E. A., & Pfennig, D. W. (2021). Evolutionary rescue via transgenerational plasticity: Evidence and implications for conservation. *Evolution & Development*, 23(4), 292-307.

Hille, S. M., & Cooper, C. B. (2015). Elevational trends in life histories: Revising the pace-of-life framework. *Biological Reviews*, 90(1), 204-213.

Howald, H., & Hoppeler, H. (2003). Performing at extreme altitude: muscle cellular and subcellular adaptations. *European journal of applied physiology*, 90(3), 360-364.

Huey RB. Temperature, physiology and the ecology of reptiles. In: Gans C, Pough FH, editors. *Biology of the Reptilia*. Academic Press, London; 1982. pp. 25–91

Huey, R. B., Kearney, M. R., Krockenberger, A., Holtum, J. A., Jess, M., & Williams, S. E. (2012). Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367(1596), 1665-1679.

Jiang, Z. W., Ma, L., Mi, C. R., & Du, W. G. (2021). Effects of hypoxia on the thermal physiology of a high-elevation lizard: implications for upslope-shifting species. *Biology Letters*, 17(3), 20200873.

Jiménez-Robles, O. & de la Riva, I. (2019). Lizards in the mist: Thermal niches constrained by habitat and microclimates in the Andes of southern Bolivia. *Journal of Biogeography* 46, 1676-1686.

Juan, C., Emerson, B. C., Oromí, P., & Hewitt, G. M. (2000). Colonization and

diversification: towards a phylogeographic synthesis for the Canary Islands. *Trends in Ecology & Evolution*, 15(3), 104-109.

Kenner, R. A. & Ahmed, S. I. (1975) Measurements of electron transport activities in marine phytoplankton. *Marine Biology*, 33, 119–127

Körner, C. (2007). The use of ‘altitude’ in ecological research. *Trends in ecology & evolution*, 22(11), 569-574.

Koziel, G.; S`khifa, A.; Kirchhof, S.; MacLeod, A.; Joger, U.; Sinervo, B.; Carretero, M. A.; Slimani, T. & Vences, M. (2021): Inferred preferred temperatures of lizards differ substantially among experimental methods. *Salamandra*, 57(2): 263-273.

Laiolo, P., & Obeso, J. R. (2017). Life-history responses to the altitudinal gradient. In *High mountain conservation in a changing world* (pp. 253-283). Springer, Cham.

Lampinen, J.; Raitio, M.; Perala, A.; Oranen, H.; Harinen, R.-R. (2012) Microplate based pathlength correction method for photometric DNA quantification assay, in: Scientific, V.S.A.A.L.T.F. (Ed.).

Leclair Jr, R., Leclair, M. H., Dubois, J., & Daoust, J. L. (2000). Age and size of wood frogs, *Rana sylvatica*, from Kuujjuarapik, Northern Quebec. *Canadian Field-Naturalist*, 114(3), 381-387.

Levis, N. A., & Pfennig, D. W. (2016). Evaluating ‘plasticity-first’ evolution in nature: key criteria and empirical approaches. *Trends in ecology & evolution*, 31(7), 563-574.

Lewis, P., Sheehan, D., Soares, R., Varela Coelho, A., & O'Halloran, K. D. (2015). Chronic sustained hypoxia-induced redox remodeling causes contractile dysfunction in mouse sternohyoid muscle. *Frontiers in physiology*, 6, 122.

Lleonart, J., Salat, J., & Torres, G. J. (2000). Removing allometric effects of body size in morphological analysis. *Journal of Theoretical Biology*, 205, 85–93.

Lorenzon, P., Clobert, J., Oppliger, A., John-Adler, H. (1999). Effect of water constraint on growth rate, activity and body temperature of yearling common lizard. *Oecologia*, 118, 423–430.

Lund, T. C., Glass, T. J., Tolar, J., Blazar, B. R. (2009). Expression of telomerase and telomere length are unaffected by either age or limb regeneration in *Danio rerio*. *PLoS ONE* 4, e7688.

Martín, J. & López, P. (1996). Avian Predation on a Large Lizard (*Lacerta lepida*) Found

at Low Population Densities in Mediterranean Habitats: An Analysis of Bird Diets. *Copeia*, 3, 722-726.

Masó, G., Kaufman, J., Clavero, H. & Fitze, P. S. (2019). Age-dependent effects of moderate differences in environmental predictability forecasted by climate change, experimental evidence from a short-lived lizard (*Zootoca vivipara*). *Scientific Reports*, 9, 15546.

Mawdsley, J. R., O'Malley, R., Ojima, D. S. (2009). A review of climate-change adaptation strategies for wildlife management and biodiversity conservation. *Conservation Biology*, 23(5), 1080-1089.

Megía-Palma R., Martínez J., & Merino S. (2016). A structural colour ornament correlates positively with parasite load and body condition in an insular lizard species. *The Science of Nature*, 103(7), 1-10.

Megía-Palma, R., Jiménez-Robles, O., Hernández-Agüero, J., & Riva, I. (2020). Plasticity of haemoglobin concentration and thermoregulation in a mountain lizard. *Journal of Thermal Biology*, 92, 102656.

Meiri, S., Bauer, A.M., Chirio, L., Colli, G.R., Das, I., Doan, T. M., Feldman, A., Herrera, F.-C., Novosolov, M., Pafilis, P., Pincheira-Donoso, D., Powney, G., Torres-Carvajal, O., & Uetz, P. (2013). Are lizards feeling the heat? A tale of ecology and evolution under two temperatures. *Global Ecology and Biogeography*, 22(7), 834-845.

Monaghan, P., Metcalfe, N. B., & Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology letters*, 12(1), 75-92.

Muñoz, M. M. (2021). The Bogert effect, a factor in evolution. *Evolution*, 76, 49-66.

Olsson, M., Wapstra, E., & Friesen, C. (2018). Ectothermic telomeres: it's time they came in from the cold. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 373(1741), 20160449.

Paaijmans, K. P., Heinig, R. L., Seliga, R. A., Blanford, J. I., Blanford, S., Murdock, C. C., & Thomas, M. B. (2013). Temperature variation makes ectotherms more sensitive to climate change. *Global change biology*, 19(8), 2373-2380.

Packard, T. 1971. The measurement of respiratory electron-transport activity in marine phytoplankton. – J. Mar. Res. 29: 235–244.

- Perry, G., Wallace, M. C., Perry, D., Curzer, H. and Muhlberger, P. 2011. Toe Clipping of Amphibians and Reptiles: Science, Ethics, and the Law. - *J. Herpetol.* 45: 547-555.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research*, 29(9), e45-e45.
- Pianka, E. R., & Vitt, L. J. (2003). *Lizards: windows to the evolution of diversity* (Vol. 5). Univ of California Press.
- Pleguezuelos, J.M.; Márquez, R.; Lizana, M. (2002). *Atlas y Libro Rojo de los anfibios y reptiles de España*. Madrid: Dirección General de Conservación de la naturaleza; Asociación Herpetológica Española; Organismo Autónomo Parques Nacionales.
- Quagliariello, A., Di Paola, M., De Fanti, S., Gneccchi-Ruscione, G. A., Martinez-Priego, L., Pérez-Villaroya, D., Sherpa, M. G., Sherpa, P. T., Marinelli, G., Natali, L., Di Marcello, M., Peluzzi, D., Di Cosimo, P. D'Auria, G., Pettener, G., Sazzini, M., Luiselli D. & De Filippo, C. (2019). Gut microbiota composition in Himalayan and Andean populations and its relationship with diet, lifestyle and adaptation to the high-altitude environment. *J Anthropol Sci*, 96, 189-208.
- R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>
- Rasmussen, R. (2001). Quantification on the LightCycler. In *Rapid cycle real-time PCR* (pp. 21-34). Springer, Berlin, Heidelberg
- Reinke, B. A., Cayuela, H., Janzen, F. J., Lemaître, J. F., Gaillard, J. M., Lawing, et al. (2022). Diverse aging rates in ectothermic tetrapods provide insights for the evolution of aging and longevity. *Science*, 376, 1459-1466.
- Rochette, P. J., & Brash, D. E. (2010). Human telomeres are hypersensitive to UV-induced DNA Damage and refractory to repair. *PLoS genetics*, 6(4), e1000926.
- Roitberg, E. S., Orlova, V., Bulakhova, N. A., Kuranova, V. N., Eplanova, G. V., Zinenko, O. I., Arribas, O. J., Kratochvíl, L., Ljubisavljevic, K. & Starikov, V. P. (2020). Variation in body size and sexual size dimorphism in the most widely ranging lizard: testing the effects of reproductive mode and climate. *Ecology and Evolution*, 10, 4531-4561.
- Rozen-Rechels, D., Dupoué, A., Lourdaïs, O., Chamaillé-Jammes, S., Meylan, S., & Clobert, J. (2019). When water interacts with temperature: ecological and evolutionary implications of thermo-hydreregulation in terrestrial ectotherms. *Ecology and Evolution*, 9(17), 10029-43.

- Rubalcaba, J. G., Verberk, W. C., Hendriks, A. J., Saris, B., & Woods, H. A. (2020). Oxygen limitation may affect the temperature and size dependence of metabolism in aquatic ectotherms. *Proceedings of the National Academy of Sciences*, 117(50), 31963-31968.
- Salvador, A. (2015). Lagarto tizón – Gallotia galloti. En: Enciclopedia Virtual de los Vertebrados Españoles. Salvador, A., Marco, A. (Eds.). Museo Nacional de Ciencias Naturales, Madrid. <http://www.vertebradosibericos.org>
- Sandercock, B. K., Martin, K. & Hannon, S. J. (2005). Life history strategies in extreme environments: comparative demography of arctic and alpine ptarmigan. *Ecology* 86, 2176–2186.
- Sannolo, M., & Carretero, M. A. (2019). Dehydration constrains thermoregulation and space use in lizards. *PLoS One*, 14(7), e0220384.
- Sannolo, M., Civantos, E., Martín, J., & Carretero, M. A. (2020). Variation in field body temperature and total evaporative water loss along an environmental gradient in a diurnal ectotherm. *Journal of Zoology*, 310(3), 221-231.
- Schulte, U., Hochkirch, A., Lötters, S., Rödder, D., Schweiger, S., Weimann, T., & Veith, M. (2012). Cryptic niche conservatism among evolutionary lineages of an invasive lizard. *Global Ecology and Biogeography*, 21, 198–211.
- Scott, N. M., Haussmann, M. F., Elsey, R. M., Trosclair, P. L., & Vleck, C. M. (2006). Telomere length shortens with body length in *Alligator mississippiensis*. *Southeast. Nat.* 5, 685–692.
- Sears, M. W. (2005). Geographic variation in the life history of the sagebrush lizard: the role of thermal constraints on activity. *Oecologia*, 143, 25–36.
- Seebacher, F., Holmes, S., Roosen, N. J., Nouvian, M., Wilson, R. S., & Ward, A. J. (2012). Capacity for thermal acclimation differs between populations and phylogenetic lineages within a species. *Functional Ecology*, 26(6), 1418-1428.
- Seebacher, F., White, C. R., & Franklin, C. E. (2015). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nature Climate Change*, 5(1), 61-66.
- Simčič, T. & Brancelj, A. 1997. Electron transport system (ETS) activity and respiration rate in five *Daphnia* species at different temperatures. – *Hydrobiology* 360: 117–125.

- Simčič, T., Pajk, F., Jaklič, M., Brancelj, A., & Vrezec, A. (2014). The thermal tolerance of crayfish could be estimated from respiratory electron transport system activity. *Journal of thermal biology*, 41, 21-30.
- Sinervo, B., Mendez-De-La-Cruz, F., Miles, D. B., Heulin, B., Bastiaans, E., Villagrán-Santa Cruz, M., Lara-Resendiz R., Martinez-Mendez N., Calderon-Espinosa M. L., Meza-Lazaro, R. N., Gadsden, H., Avila, L., Morando, M., De La Riva, I., Sepulveda, P., Rocha, C., Ibarquengoytia, N., Puntriano, C., Massot, M., Lepetz, V, et al. & Sites, J. (2010). Erosion of lizard diversity by climate change and altered thermal niches. *Science*, 328(5980), 894-899
- Solano, L. A. R., Navas, C. A., Carvajalino-Fernández, J. M., & Amézquita, A. (2016). Thermal ecology of montane *Atelopus* (Anura: Bufonidae): A study of intrageneric diversity. *Journal of thermal biology*, 58, 91-98.
- Storz, J. F., & Scott, G. R. (2019). Life ascending: mechanism and process in physiological adaptation to high-altitude hypoxia. *Annual review of ecology, evolution, and systematics*, 50, 503-526.
- Turbill, C., Bieber, C. & Ruf, T. (2011). Hibernation is associated with increased survival and the evolution of slow life histories among mammals. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **278**, 3355–3363
- Veríssimo, C. V., & Carretero, M. A. (2009). Preferred temperatures of *Podarcis vaucheri* from Morocco: intraspecific variation and interspecific comparisons. *Amphibia-Reptilia*, 30(1), 17-23.
- Wang Y, Gao Z, Shu Z, Li R, Huai J, Wei B, et al. (2016) Low precipitation aggravates the impact of extreme high temperatures on lizard reproduction. *Oecologia*. 2016; 182:961–971.
- Western, D. & Ssemakula, J. (1982). Life-history patterns in birds and mammals and their evolutionary interpretation. *Oecologia* 54, 281–290
- Whittemore, K., Vera, E., Martínez-Nevado, E., Sanpera, C., & Blasco, M. A. (2019). Telomere shortening rate predicts species life span. *Proceedings of the National Academy of Sciences*, 116(30), 15122-15127.
- Žagar, A., Carretero, M. A., Osojnik, N., Sillero, N., & Vrezec, A. (2015). A place in the sun: interspecific interference affects thermoregulation in coexisting lizards. *Behavioral Ecology and Sociobiology*, 69(7), 1127-1137.

Žagar, A., Carretero, M. A., Marguč, D., Simčič, T., & Vrezec, A. (2018a). A metabolic syndrome in terrestrial ectotherms with different elevational and distribution patterns. *Ecography*, 41(10), 1728-1739.

Žagar, A., Holmstrup, M., Simčič, T., Debeljak, B., & Slotsbo, S. (2018b). Comparing *Enchytraeus albidus* populations from contrasting climatic environments suggest a link between cold tolerance and metabolic activity. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 224, 35-41.

Žagar, A., Simčič, T., Dajčman, U., & Megía-Palma, R. (2022): Parasitemia and elevation as predictors of hemoglobin concentration and antioxidant capacity in two sympatric lizards. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 270, 111233.

Zamocky, M., Furtmüller, P.G., & Obinger, C. (2008). Evolution of catalases from bacteria to humans. *Antioxid. Redox Signal.* 10, 1527–1548.

Zamora-Camacho, F. J., Reguera, S., Moreno-Rueda, G., & Pleguezuelos J. M. (2013). Patterns of seasonal activity in a Mediterranean lizard along a 2200 m altitudinal gradient. *Journal of Thermal Biology*, 38 (2), 64-69.

2.8. Supplementary materials

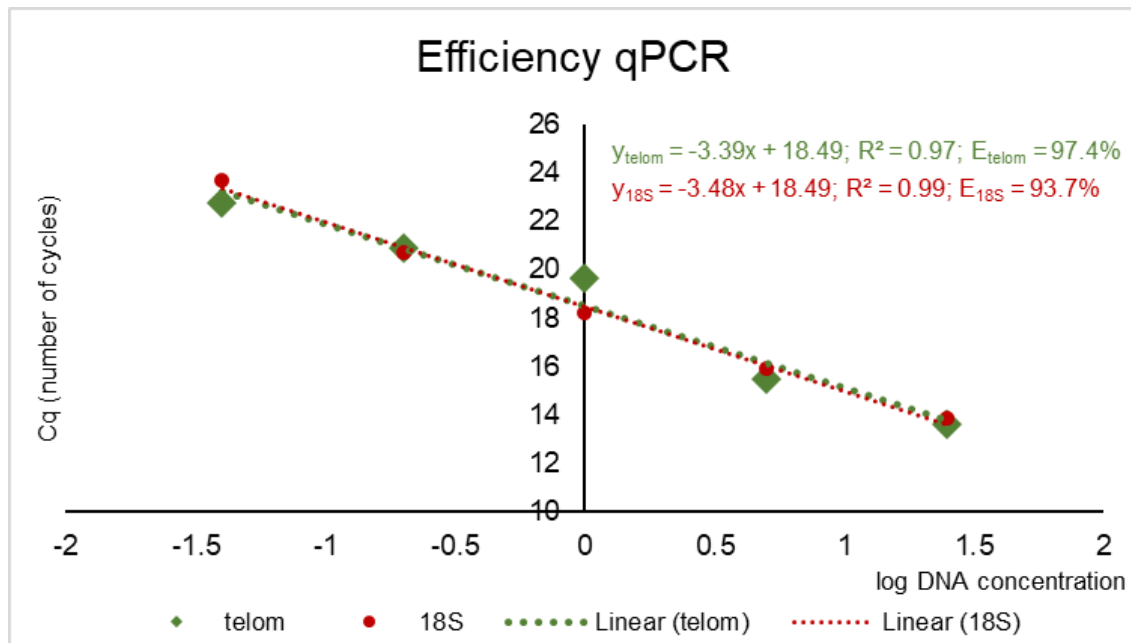


Figure S2.1. – Determination of real-time qPCR efficiencies (mean of all 96-well plates) of the reference gene (18S) and the telomere amplified fragments (telom). The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ (Rasmussen, 2001).

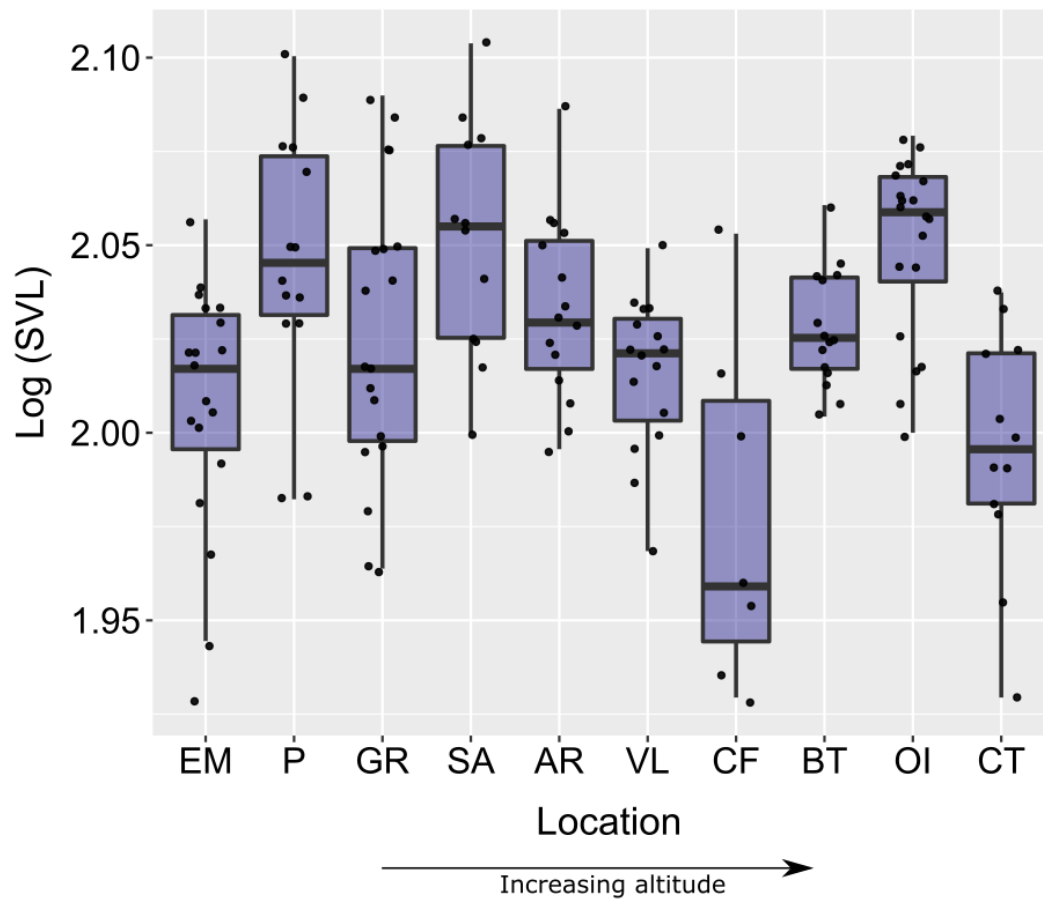


Figure S2.2. Boxplot showing variation of size in the form of SVL (snout-vent length) using a log10 transformation along locations which are ordered by increasing altitudes from 100m to 3550m.

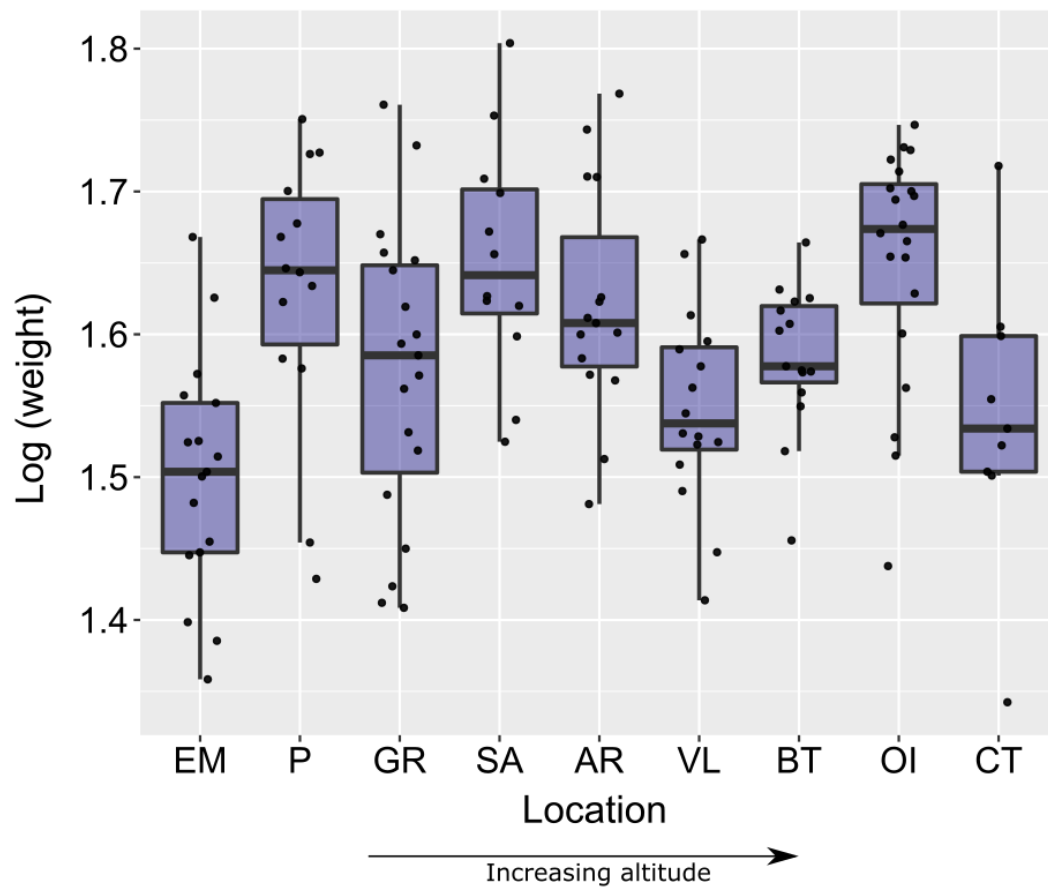


Figure S2.3. Boxplot showing variation of weight using a log10 transformation along locations which are ordered by increasing altitudes from 100m to 3550m.

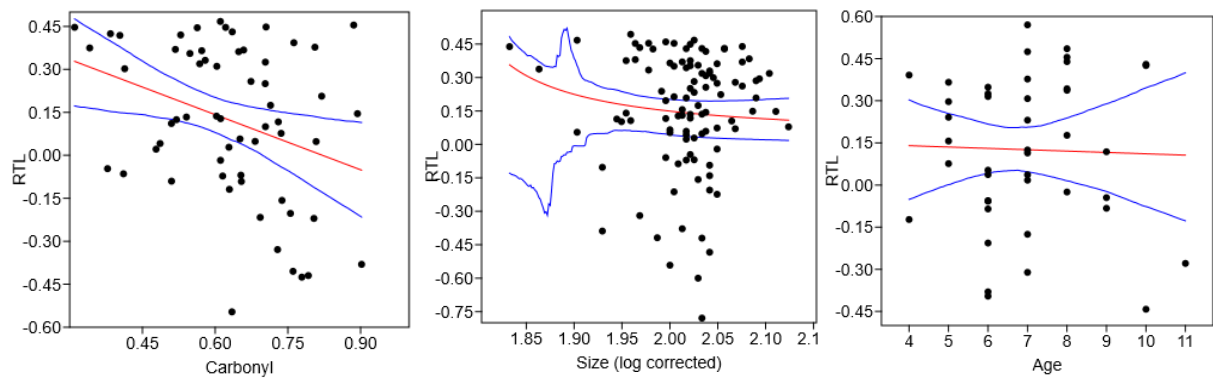


Figure S2.4 - Plotted correlations of RTL: carbonyl, age and size (snout vent length; SVL). RTL and carbonyl controlled for size and log-transformed and size was only log-transformed

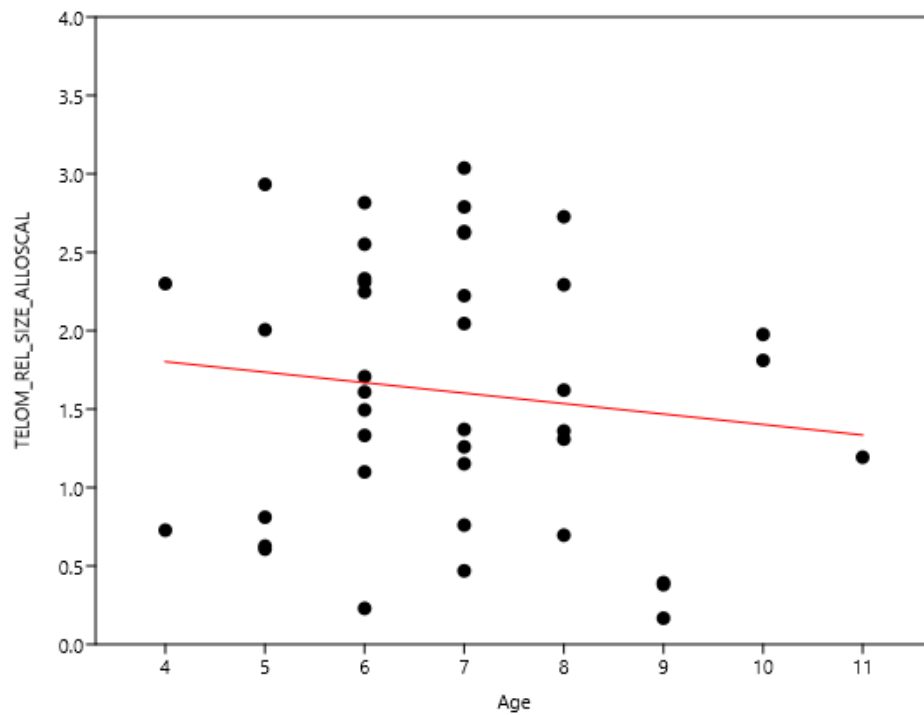


Figure S2.5. Plotted correlation between skeletochronological age estimation and log-transformed relative telomere length (RTL).

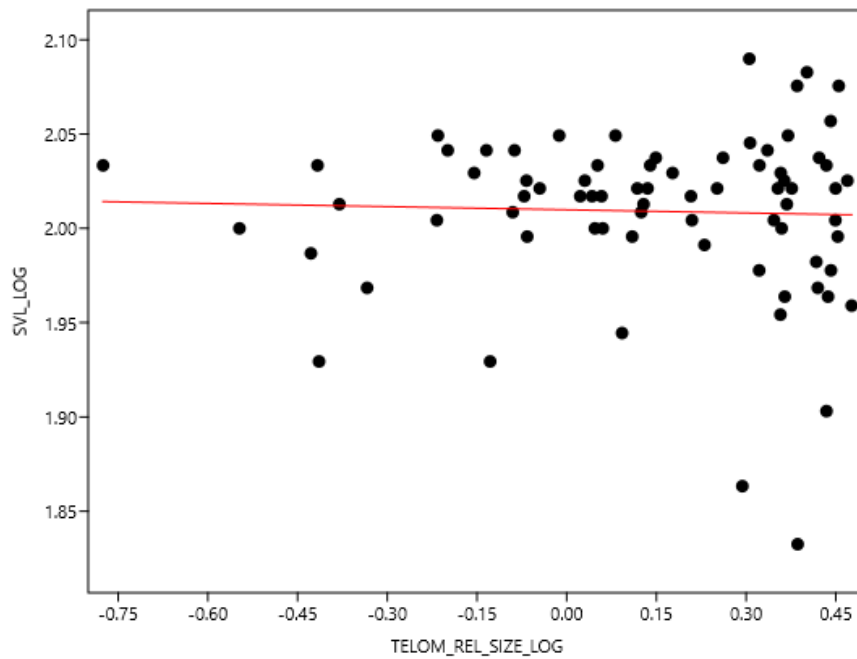


Figure S2.6. Plotted correlation between log-transformed snout-vent length (SVL) and log-transformed relative telomere length (RTL).

Table S2.1. Standard statistics for all non-transformed studied variables. Abbreviations: SVL – Snout-vent length; T_p - preferred temperature; EWL_i - instantaneous evaporative water loss; EWL_a - accumulated evaporative water loss; PMA - potential metabolic activity; RTL - relative telomere length. * All repeated measures; ** only last EWL_a measure

Variables	Median	Mean	SD	Min	Max
Age (years)	7.00	7.03	1.57	4	11
SVL (mm)	105.00	103.22	8.42	73	121
Weight (g)	36.08	36.36	7.01	22.83	57.65
T_p (°C)*	33.84	33.95	1.82	23.1	39.7
EWL_i (g) *	0.0006	0.0007	0.0006	0.00001	0.0062
EWL_a (g) **	0.0075	0.0080	0.0040	0.0027	0.0184
PMA (μLO_2 /mg prot/h) *	5.01	5.20	2.18	1.44	12.77
Catalase (U/mg protein)	6.70	7.34	2.91	2.66	16.55
Carbonyl (nmole/mg protein)	4.51	4.57	1.37	2.14	7.97
RTL	1.58	1.63	0.76	0.17	3.00

Table S2.2. - Telomere efficiency qPCR amplification calibrator sample reaction tests. Cq is the quantification cycle value.

DNA concentration (ng)	Telomeres Cq (number of cycles)	18S Cq (number of cycles)	Log (DNA concentration)
0.04	22.78	23.67	-1.398
0.2	20.87	20.71	-0.699
1	19.67	18.25	0
5	15.47	15.91	0.699
25	13.65	13.90	1.398

Table S2.3. Same as Table 2.1 from the current chapter, but sample numbers have already been filtered for statistical analysis (after outlier removal or non-validated age estimation score for this variable). Abbreviations: SVL – Snout-vent length; T_p - preferred temperature; EWL_i - instantaneous evaporative water loss; EWL_a - accumulated evaporative water loss; PMA - potential metabolic activity; RTL - relative telomere length.

Altitude (m)	150	600	1,300	1,900	2,350	2,750	2,900	100	600	1,350	2,300	3,550,
Location	P	SA	AR	CF	OI	TSE	TRE	EM	GR	VL	BT	CT
Age	11	7	11	8	11	8	6	14	13	10	8	8
T_p	-	-	-	-	-	-	-	14	16	13	14	6
EWL_i	-	-	-	-	-	-	-	12	17	15	15	6
EWL_a	-	-	-	-	-	-	-	15	18	16	15	6
PMA	-	-	-	-	-	-	-	15	17	17	13	7
Catalase	-	-	-	-	-	-	-	15	17	17	12	7
Carbonyl	-	-	-	-	-	-	-	13	17	17	13	6
RTL	-	-	-	-	-	-	-	14	19	16	14	11

Table S2.4 - SVL pairwise differences using Benjamini-Hockberg *P*-value correction; in bold are significant *P* values

LOC1	LOC2	<i>P</i> value	<i>P</i> value (adjustment)
EM	P	<0.001	0.002
EM	GR	0.054	0.090
P	GR	0.064	0.103
EM	SA	<0.001	0.001
P	SA	0.662	0.710
GR	SA	0.026	0.053
EM	AR	0.014	0.031
P	AR	0.249	0.321
GR	AR	0.510	0.574
SA	AR	0.122	0.167
EM	VL	0.416	0.480
P	VL	0.007	0.016
GR	VL	0.300	0.355
SA	VL	0.002	0.007
AR	VL	0.108	0.152
EM	CF	0.030	0.056
P	CF	<0.001	<0.001
GR	CF	<0.001	0.002
SA	CF	<0.001	<0.001
AR	CF	<0.001	0.001
VL	CF	0.007	0.016
EM	BT	0.053	0.090
P	BT	0.101	0.146
GR	BT	0.899	0.919
SA	BT	0.044	0.080
AR	BT	0.615	0.675
VL	BT	0.271	0.335
CF	BT	<0.001	0.002
EM	OI	<0.001	<0.001
P	OI	0.697	0.730
GR	OI	0.014	0.031
SA	OI	0.921	0.921
AR	OI	0.100	0.146
VL	OI	0.001	0.003
CF	OI	<0.001	<0.001
BT	OI	0.030	0.056
EM	CT	0.276	0.335
P	CT	<0.001	<0.001
GR	CT	0.006	0.016
SA	CT	<0.001	<0.001
AR	CT	0.001	0.005
VL	CT	0.077	0.119
CF	CT	0.234	0.310
BT	CT	0.006	0.016
OI	CT	<0.001	<0.001

Table S2.5 - Weight pairwise differences using Benjamini-Hockberg *P*-value correction; in bold are significant *P* values

LOC1	LOC2	<i>P</i> value	<i>P</i> value (adjustment)
EM	P	<0.001	<0.001
EM	GR	0.012	0.033
P	GR	0.050	0.094
EM	SA	<0.001	<0.001
P	SA	0.529	0.595
GR	SA	0.012	0.033
EM	AR	<0.001	0.001
P	AR	0.747	0.791
GR	AR	0.097	0.174
SA	AR	0.343	0.441
EM	VL	0.109	0.186
P	VL	0.008	0.026
GR	VL	0.395	0.474
SA	VL	0.002	0.008
AR	VL	0.017	0.041
EM	BT	0.006	0.023
P	BT	0.129	0.211
GR	BT	0.708	0.772
SA	BT	0.037	0.074
AR	BT	0.222	0.341
VL	BT	0.246	0.341
EM	OI	<0.001	<0.001
P	OI	0.493	0.572
GR	OI	0.004	0.016
SA	OI	0.981	0.981
AR	OI	0.294	0.392
VL	OI	<0.001	0.003
BT	OI	0.020	0.044
EM	CT	0.236	0.341
P	CT	0.015	0.037
GR	CT	0.374	0.464
SA	CT	0.004	0.016
AR	CT	0.028	0.059
VL	CT	0.864	0.888
BT	CT	0.247	0.341
OI	CT	0.002	0.008

Table S2.6 – Ordinary Least Squares Regression of age and log-transformed size (snout-vent length; SVL).

	Sum of Squares	df	MS	F	<i>P</i> value
Regression	0.087	1	0.087	71.801	<0.001
Residual	0.136	113	0.001		
Total Sum of Squares	0.223				

Table S2.7. – Pairwise differences tests on EWL_a using Benjamini-Hockberg P -value correction.

LOC1	LOC2	P value	P value (adjustment)
EM	GR	0.002	0.003
EM	VL	0.911	0.911
GR	VL	0.002	0.003
EM	BT	<0.001	<0.001
GR	BT	0.070	0.100
VL	BT	<0.001	<0.001
EM	CT	0.002	0.003
GR	CT	0.335	0.419
VL	CT	0.001	0.003
BT	CT	0.710	0.789

Table S2.8. – Pairwise differences tests on EWL_i using Benjamini-Hockberg P -value correction.

LOC1	LOC2	P value	P value (adjustment)
EM	GR	0.053	0.089
EM	VL	0.486	0.486
GR	VL	0.195	0.195
EM	BT	<0.001	<0.001
GR	BT	0.003	0.008
VL	BT	<0.001	<0.001
EM	CT	0.006	0.014
GR	CT	0.166	0.238
VL	CT	0.021	0.042
BT	CT	0.389	0.432

Table S2.9. – Pairwise differences tests on PMA using Benjamini-Hockberg *P*-value correction.

LOC1	LOC2	<i>P</i> value	<i>P</i> value (adjustment)
EM	GR	0.010	0.019
EM	VL	0.101	0.168
GR	VL	0.195	0.356
EM	BT	0.291	0.356
GR	BT	<0.001	0.003
VL	BT	0.008	0.192
EM	CT	0.167	0.239
GR	CT	0.001	0.003
VL	CT	0.007	0.019
BT	CT	0.620	0.620

Table S2.10 - Catalase antioxidant activity pairwise differences with non-pooled SD using Benjamini-Hockberg *P*-value correction.

LOC1	LOC2	<i>P</i> value	<i>P</i> value (adjustment)
EM	GR	0.102	0.510
EM	VL	0.256	0.640
GR	VL	0.943	0.970
EM	BT	0.029	0.290
GR	BT	0.474	0.819
VL	BT	0.562	0.819
EM	CT	0.172	0.573
GR	CT	0.650	0.819
VL	CT	0.655	0.819
BT	CT	0.970	0.970

Table S2.11 - Carbonyl metabolites pairwise differences with non-pooled SD using Benjamini-Hockberg *P*-value correction; in bold are significant *P* values

LOC1	LOC2	<i>P</i> value	<i>P</i> value (adjustment)
EM	GR	0.166	0.277
EM	VL	0.055	0.216
GR	VL	0.670	0.744
EM	BT	0.087	0.216
GR	BT	0.979	0.979
VL	BT	0.652	0.744
EM	CT	0.005	0.046
GR	CT	0.112	0.244
VL	CT	0.237	0.339
BT	CT	0.083	0.216

Table S2.12 – RTL pairwise differences with non-pooled SD; using Benjamini-Hockberg *P*-value correction; in bold are significant *P* values

LOC1	LOC2	<i>P</i> value	<i>P</i> value (adjustment)
EM	GR	0.425	0.607
EM	VL	0.214	0.428
GR	VL	0.561	0.638
EM	BT	0.410	0.607
GR	BT	0.979	0.979
VL	BT	0.574	0.638
EM	CT	0.014	0.051
GR	CT	0.015	0.051
VL	CT	0.052	0.129
BT	CT	0.015	0.051

Table S2.13 – Ordinary Least Squares Regression of: Carbonyl and RTL (relative telomere length), Age and RTL, and size (snout vent length; SVL) and RTL log transformed. Variables are log transformed and controlled for size except for age and size that are only log transformed

		Sum of Squares	df	MS	F	<i>P</i> value
Carbonyl vs RTL	Regression	0.453	1	0.424	6.4291	0.0139
	Residual	3.821	58	0.0659		
	Total Sum of Squares	4.244				
Age vs. RTL	Regression	0.0311	1	0.0311	0.310	0.419
	Residual	3.914	39	0.100		
	Total Sum of Squares	3.945				
SVL vs. RTL	Regression	0.0029	1	0.0029	0.0403	0.159
	Residual	7.309	101	0.0724		
	Total Sum of Squares	7.3121				

Chapter III – Selection on hypoxia-associated genes mediates altitudinal adaptation in an insular lizard

Selection on hypoxia-associated genes mediates altitudinal adaptation in an insular lizard

Nina Serén^{1,2,3}, Pedro Andrade^{1,2}, Rodrigo Megía-Palma^{1,2,4}, Maria Magalhães^{1,2}, Miguel Carneiro^{1,2}, Anamarija Žagar^{1,2,5}, Catarina Pinho^{1,2}, Miguel A. Carretero^{1,2,3}

¹CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal

²BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal

³Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, 4099-002 Porto, Portugal

⁴Universidad de Alcalá (UAH), Parasitology Unit, Department of Biomedicine and Biotechnology, School of Pharmacy, Spain

⁵Department of Organisms and Ecosystems Research, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia

3.1. Abstract

High altitude imposes several extreme constraints on life, such as low oxygen pressure and high levels of ultraviolet radiation, which require specialised adaptations. Many studies have focused on how endothermic vertebrates respond to these challenging environments, but there is still much uncertainty on the genomic mechanisms employed by ectotherms in similar conditions. In this study we used whole-genome sequencing of low-altitude (100-600 m) and high-altitude (3,700 m) populations of the insular lizard *Gallotia galloti* to uncover signatures of altitudinal adaptation. We demonstrate that the studied populations are largely undifferentiated and mostly share similar patterns of genetic variation. Selective sweep mapping suggests that signatures of adaptation to high-altitude are not widespread across the genome, clustering in a relatively small number of genomic regions. Of particular importance, the two most striking outlier regions were located in the vicinity of genes associated with response to hypoxia, in particular genes linked to hypoxia-induced pulmonary vascular growth. These results underline how low oxygen pressure generates the most consistent selective constraint in high altitude environments, to which vertebrates with vastly contrasting physiological profiles need to adapt in a context on ongoing climate change.

Key words: genomics, *Gallotia galloti*, whole-genome sequencing, high-altitude, hypoxia

3.2. Introduction

High altitude habitats are among the harshest environments for living organisms. Temperature extremes, high ultraviolet radiation and low oxygen pressure are unique features of these environments when compared to lowland areas (Blumthaler et al. 1997; Jørgensen & Fath, 2008), and these abiotic characteristics have been found to drive adaptation in highland plant and animal populations at any latitude (Monge & León-Velarde, 1991; Simonson et al., 2010; Halbritter et al., 2018; Storz & Scott, 2019; Wang et al., 2021). In addition to this natural challenge, ongoing anthropogenic environmental disturbances that include climatic changes (Jørgensen & Fath, 2008; Thornton et al., 2014) have the potential to interact with the constraints placed by high altitude, not only for altitudinal specialists but also for lowland species. For example, higher temperatures are driving some lowland species to shift their distribution towards higher altitudes, where lower temperatures can be experienced, but in turn forcing them to cope with additional constraints on their novel physical environments (Jiang et al., 2021). Coping with these multiple stressors at very high altitudes requires a suite of behavioural and physiological adaptations, including optimization of the respiratory process (decrease in mitochondrial density, larger lungs or alveolar structures, haemoglobin with higher O₂ affinity), thermoregulatory or hydric adjustments (changes of size, shape, colouration, physiology or behaviour), or differentiation of gut microbiota as a response to changed diet (Howald & Hoppeler, 2003; Solano et al., 2016; Souchet et al., 2020; González-Morales et al., 2021; Žagar et al., 2018, 2022; Quagliarello et al., 2019; Storz & Scott, 2019; Sannolo et al., 2020; S'khifa et al., 2022). However, only recently have we started gaining insights into the evolutionary mechanisms that mediate these responses.

Studies on the genomics of adaptation to altitude have greatly advanced in recent years. These have mostly been driven by research done on endothermic vertebrates, for which multiple studies have found links between specific genes and adaptation to altitude. For example, alleles of the gene Endothelial PAS Domain Protein 1 (*EPAS1*) have been repeatedly implicated in adaptation to altitude in humans (Yang et al., 2017a; Huerta-Sánchez et al., 2014), deer mice (Schweizer et al., 2019), horses (Hendrickson, 2013; Liu et al., 2019), goats (Song et al., 2016), dogs (Gou et al., 2014; Fan et al., 2017) and snakes (Li et al., 2018). In these species, this gene is responsible for regulating haemoglobin concentration and angiogenesis (development of new blood vessels), which makes it a prime target for selection in environments with low atmospheric oxygen pressure. Several other genes have also been linked to altitudinal adaptation, mostly hypoxia-related (e.g. Malik et al., 2012; Chiou et al., 2022 and see also list in table 1 in

Witt et al., 2019) but also genes associated with metabolism and cold resistance (Cheviron et al., 2008, Velotta et al., 2020), skeletal development (Qu et al., 2013; Wang et al., 2014) and DNA repair (Yang et al., 2014; Zhang et al., 2016; Li et al., 2018) showing the complex nature of adaptation to an environment with multiple variables and constraints (reviewed by Storz, 2021).

Since ectotherms have restricted homeostatic mechanisms, they are more likely to be directly exposed to temperature or precipitation shifts (Angilletta, 2009), that vary greatly with altitude. In this context, ectothermic vertebrates are key models to understand how apparently vulnerable species can respond to the challenges of environmental change (Sinervo et al., 2010; Paaïmans et al., 2013). Heliothermic ectotherms, such as lizards, are able to keep body temperature close to physiological optimal to be fully active. While behavioural thermoregulation may substantially decrease local environmental pressures on lizard thermal physiology (Muñoz, 2021), basking exposes them to the harmful effects of ultraviolet radiation or extreme temperature variation present at great altitudes that imposes more precise thermoregulation (e.g. Navas, 1997; Solano et al., 2016; Reguera et al., 2014; González-Morales et al., 2021; Osojnik et al., 2013). The evolutionary pathways or genetic mechanisms associated with such adaptive phenotypes in ectotherms are, however, still poorly understood. Comparative genomic studies on frogs (Sun et al., 2018) and snakes (Li et al., 2018) have highlighted the roles of genes involved with DNA repair, energy metabolism and response to hypoxia when comparing pairs of species inhabiting low and high-altitude habitats. Transcriptomic studies on frogs (Yang et al., 2016, Yang et al., 2017b), on the other hand, have not found evidence for a role of genes associated with hypoxia response. To help disentangle the effects of phylogenetic signal and context-dependent gene expression in adaptation, species that cover a wide range of altitudinal habitats with minimal genetic structure are needed as models for genetic association studies.

The Tenerife lizard, *Gallotia galloti* (Figure 3.1a) is ideally placed to answer questions on the evolution of altitudinal adaptation in ectotherms. This species, an endemic to the La Palma and Tenerife islands in the Canary Islands archipelago (Figure 3.1b), is thought to have colonized the paleoislands of Tenerife between 2 to 4 My ago (Cox et al., 2010), which were joined into the Tenerife island we know today through multiple volcanic events that started around 2 My ago (Ancochea et al., 1990). Previous studies on the genetic structure found little to moderate differentiation between populations of *G. galloti* within Tenerife (Richard & Thorpe, 2001; Thorpe & Richard, 2001; Brown et al., 2016). Populations inhabiting the southern slope of the island are the

only *Gallotia* that can be found continuously from the sea level all the way to the summit of the island's volcanic cone (Fariña et al., 2011). This volcanic cone, which starts at an altitude of approximately 2,300 metres, has grown in the period between 170,000-30,000 years ago, with its last volcanic eruption occurring nearly 1,300 years ago (Carracedo et al., 2007). This eruptive history together with recent glaciations (around 30,000 years ago) that likely covered high altitudes with snow, suggests a relatively recent colonization of *G. gallotia* of the summit of the volcano.

Here we used whole-genome sequencing to study adaptation to extreme altitudinal environments in the southern populations of *G. galloti*. We assembled the genome of this species *de novo* using linked-read sequencing, and then re-sequenced DNA pools of high and low-altitude populations of *G. galloti* to examine the patterns of genetic variation. With this approach, our main objective was to find regions of the genome with evidence of selection on high-altitude populations relative to low-altitude, and assess potential links to genes with relevant functions for coping with the constraints of high-altitude environments.

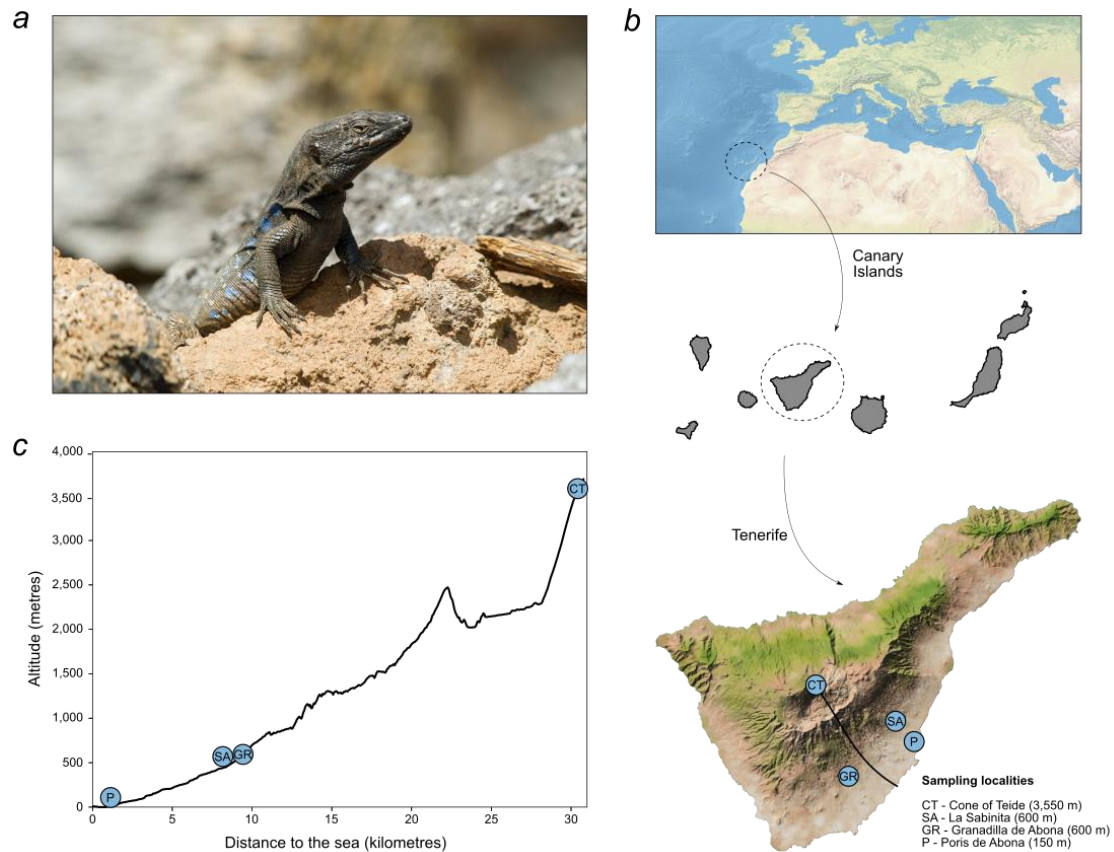


Figure 3.1. Altitudinal sampling design to study adaptation in an insular lizard. (a) The study species, *Gallotia galloti*, adult male. Photo by Jörg Hempel (used under a CC BY-SA 3.0 DE license). (b) Geographical location of the Canary Islands archipelago and the island of Tenerife (with labelled sampling sites for whole-genome sequencing). The solid black line drawn on the south side of the island indicates the topographic profile in the following panel. The broader map of Europe and North Africa was taken from Natural Earth (<https://www.naturalearthdata.com>). (c) Location of sampling sites along an example topographic profile of the south side of the island of Tenerife.

3.3. Methods

Field sampling

Samples of *Gallotia galloti* were collected in two field missions held between July and August of 2017 and 2018, in four different locations in the island of Tenerife, including high and low-altitude sites (Figure 3.1b, Figure 3.1c). For high-altitude sampling, lizards were collected at the summit of the island, at an altitude of approx. 3,550 metres (population CT, 28°16'12"N, 16°38'20.4"W; n = 14). For low-altitude sampling, we collected lizards at three localities: Porís de Abona (population P, approx. 150 meters, 28°9'52.8"N, 16°27'0.6"W; n = 12), Granadilla de Abona (population GR, approx. 600 meters, 28°7'16.1"N, 16°34'21.4"W; n = 16) and La Sabinita (population SA, approx. 600 meters, 28°10'58.4"N, 16°29'54.9", n = 12). This sampling strategy was focused on the extremes of the altitudinal distribution to maximize the chance to find potential allele frequency differences associated with altitude. An additional individual from Parque Nacional del Teide (28°15'18.7"N, 16°37' 17.3"W) was collected for reference genome assembly. Lizards were caught either by pitfall traps using tomato and banana as bait or by noose. Only males were sampled to maximize coverage in scaffolds derived from the Z chromosome. Biological samples consisted of tail tip tissue, minimizing disturbance (García-Muñoz et al., 2011) and taking advantage of the lizards' fast regeneration capacity (Fernández-Rodríguez et al., 2021). Moreover, < 50µL of blood was extracted from the caudal tail vein with sterile needles (BD microlance 3; 23G; 0.6 × 25 mm) from a previously ethanol-disinfected skin area and more than 2 cm away from the cloaca to avoid hemipenises. Samples were immediately stored in 96% ethanol until DNA extraction. Lizards were released in the same location of trapping.

Reference genome assembly

To assemble the genome of *G. galloti de novo* Chromium linked read technology from 10X Genomics was used (Weisenfeld et al., 2017). Muscle tissue from a single male was sent to the Genomics Services Laboratory of the HudsonAlpha Institute for Biotechnology, where genomic DNA was extracted using the MagAttract HMW DNA Kit (QIAGEN). Following fragment size evaluation, a Chromium library was prepared following the manufacturer's protocol, and sequenced on a NovaSeq instrument (Illumina). This generated a total of 600.03 million reads. To assemble the genome, *Supernova* v2.1.1 (Weisenfeld et al., 2017) was used with the full read dataset (raw

coverage of 69.28X) and default options. An additional assembly was trialled with a reduced number of reads (raw coverage ~35X) but this resulted in an assembly with a large number of duplicate scaffolds, so this was discarded. Additionally, upon submission to NCBI's reference genome portal, a large number of very small duplicated scaffolds were detected, so we retained only one in each pair of duplicated sequences.

To compute descriptive statistics for the assembly the script `assemblathon_stats.pl` (<https://github.com/KorfLab/Assemblathon>, Bradnam et al., 2013) was used, and genome completeness was assessed by assessing the percentage of complete single copy orthologues of the tetrapoda_odb10 database using *BUSCO* v5.3.2 (Simão et al., 2015). In addition, descriptive statistics were also computed for other lacertid genomes for comparative purposes (Kolora et al., 2019; Andrade et al., 2019; Yurchenko et al., 2020; rLacAgi1.pri: <https://www.ncbi.nlm.nih.gov/refseq/>).

Genome annotation

To annotate the *de novo* assembly, the online semi-automated pipeline from *GenSAS* v6.0 (<https://www.gensas.org>, Humann et al., 2019) was used, implementing a series of eukaryotic annotation resources. Initially, three libraries of predicted repeats were generated, one with *RepeatModeler* v2.0.1 (Flynn et al., 2020) with default settings and two with *RepeatMasker* v4.1.1 (Smit et al., 2013), using either the default GC content matrix and a 42-44% GC content matrix. The three libraries were combined to generate consensus library of repeats to mask the genome assembly. Gene prediction was performed using *Augustus* v3.3.1 (Stanke et al., 2006) with default options and using the chicken (*Gallus gallus*) to model gene structure. Additional gene prediction runs were performed using software available in *GenSAS*, with non-default *Augustus* options and through a combination of several sources of evidence with *EvidenceModeler*. However, since the *Augustus* run with default setting obtained the highest *BUSCO* score of all approaches (see “Results”), this was retained as our gene set. Gene set refinement was performed with *PASA* v2.11.0 (Haas et al., 2008). Functional annotation of the consensus gene models was done via *BLAST* against the SwissProt database, with a BLOSUM62 matrix and remaining settings as default.

Whole-genome resequencing

From each sample from our four main sites, genomic DNA was extracted from blood or tissue from tail tips using an EasySpin Genomic DNA Tissue Kit (Citomed), followed by a RNase A digestion step. DNA purity and concentration was assessed by three different methods: electrophoresis with 1% agarose gel, spectrophotometry (Nanodrop) and fluorometric quantification (Qubit dsDNA BR Assay Kit, ThermoScientific). DNA from each individual was pooled by locality in equimolar concentrations for genomic library preparation. Individual paired-end libraries were generated using Illumina's Nextera XT Kit (Illumina, San Diego, IL, USA) following the manufacturer's protocol. The libraries were quantified by qPCR using the KAPA Library Quantification Kit and pooled. Sequencing was performed at Novogene UK using a NovaSeq instrument (Illumina) using 2x150bp reads.

Read filtering and mapping

Quality control for the reads was evaluated using *FastQC* v0.11.8 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). *Trimmomatic* v.0.39 (Bolger et al. 2014) was used to remove adapter sequences, primers and low-quality reads with the following parameter settings: TRAILING:15, SLIDINGWINDOW:4:20 and MINLEN:30. Reads were then mapped to the *Gallotia galloti de novo* reference assembly with *BWA-MEM* (Li, 2013) using default settings. To maximize sequencing depth, all trimmed reads were mapped, including paired and unpaired reads. PCR duplicates were removed using Picard (<http://broadinstitute.github.io/picard/>). Mapping statistics were calculated using *SAMtools* v.0.1.19 (Danecek et al., 2021) and custom scripts.

Population genomics

To infer global population genetic parameters from the four populations summary statistics were calculated based on allele count data to call single-nucleotide polymorphisms (SNP). To assess patterns of differentiation between populations, the fixation index (F_{ST}) was obtained using *PoPoolation2* v.1.201 (Kofler et al., 2011a) in non-overlapping windows of 100 kb, restricting to positions with a minimum coverage of 7, maximum coverage of 100, minimum count of the minor allele of 3 to consider a position as a SNP, and excluding windows for which less than 20% of the window meets the coverage criteria. To calculate per-population levels and patterns of genetic variation

calculated nucleotide diversity (π , Nei, 1987) and Tajima's D (Tajima, 1989) were calculated using *PoPoolation* v1.1.2 (Kofler et al., 2011b). For both statistics, calculations were performed in non-overlapping windows of 100 kb, restricting to positions with a minimum coverage of 7, maximum coverage of 100, minimum count of the minor allele of 2 to consider a position as a SNP, and excluding windows for which less than 20% of the window meets the coverage criteria.

Selective sweep mapping

To detect regions of the genome potentially under selection in high-altitude populations, levels and patterns of genetic variation were compared along the genome. Given the main objective of comparing patterns between high and low-altitude populations, the aligned reads were merged from the three low-altitude pools to increase overall depth of coverage. To assess local patterns of genetic differentiation, F_{ST} was calculated in windows along the genome using *PoPoolation2*. Apart from differentiation, selective sweeps are also likely to lead to localized distortions in the allele frequency spectrum, such as reduction in genetic diversity and an excess of rare alleles. To test for this, *PoPoolation* provided the π and Tajima's D (TD) scores for both the high-altitude pool and the merged low-altitude pool. To highlight specifically selective sweeps acting in the high-altitude population, the mathematical formulae of π -ratio ($\pi_{\text{low-altitude}} / \pi_{\text{high-altitude}}$) and ΔTD ($TD_{\text{low-altitude}} - TD_{\text{high-altitude}}$) applied by Gazda et al. (2018) were used in our work. By setting the comparisons this way, the results were polarized to highlight recent selective sweeps in high-altitude populations. In all these analyses, the following parameters were set to minimum base quality of 30, minimum coverage of 10, maximum coverage of 100, minimum count of the minor allele of 3 to consider a position as a SNP, and excluding windows for which less than 20% of the window meets the coverage criteria. Window size was set to 50 kb, run in steps of 12.5 kb. Other window sizes were also inspected (10 kb, 100 kb), but results were qualitatively unchanged, so they are omitted here. Scaffolds smaller than 500 kb were excluded from the analyses (the largest 54 scaffolds were used, accounting for 97.4% of the total assembly size).

The three statistics used above capture different properties of sequence data under selection, which means that combining them should increase the robustness of inference on selection. To combine the independent statistics, a de-correlated composite of multiple signals was implemented (DCMS; Ma et al., 2015). DCMS was used because it considers the correlational structure of the variables to weigh their relative contributions to the combined score. Following the procedure from Gazda et al. (2018) each value was

first converted into fractional ranks to create uniformly distributed probabilities, to which an inverse-normal transformation was applied. Normalized scores were transformed into Z-scores, and from these *P*-values were calculated assuming a normal distribution of the data. Spearman's rank correlation coefficient was calculated for each pair of variables. Finally, correlation coefficients and *P*-values were used to calculate the DCMS score for each 50 kb window (taken from the previous analyses). We considered the top 0.1% of DCMS scores (as in Gazda et al., 2018) as stronger evidence of selection.

Functional annotation of variants in regions under selection

To explore the potential functional impact of variants within the regions identified as most likely under selection, we started by using *SAMtools* to extract reads from the high-altitude pool and the low-altitude merged pool for these specific regions (see Table 3.1, under Results), and used these reads to gather allele counts. These counts were used as input for the program *Pool/SNP* (Kapun et al., 2020) to conduct localized variant calling, with the following parameters: minimum coverage 10, maximum coverage of the 95% percentile of the distribution, minimum count of the alternative allele of 3, minimum frequency of 0.001, minimum base quality of 30 and discarding positions with missing data in one of the pools. Resulting VCF files were annotated using *SnpEff* v5.1 (Cingolani et al., 2012). We then used *PoPoolation2* to calculate differences in allele frequency for these annotated variants based on allele counts, using the following parameters: minimum base quality of 30, minimum coverage of 10, maximum coverage of 100 and minimum count of the minor allele of 3 to consider a position as a SNP. Detailed inspection of sequences mapping within candidate regions and allele counts was performed using *IGV* v2.12.3 (Thorvaldsdóttir et al., 2013).

3.4. Results

Assembly and annotation of the Gallotia galloti genome

To assemble the genome of *G. galloti* we used linked-read sequencing, which yielded a 1.18 Gb reference (Supplementary Figure S3.1, Supplementary Table S3.1). The genome has a high degree of scaffolding, with a scaffold N50 of 58.15 Mb. This can be considered a near-chromosome assembly since the karyotype of this species has 20 pairs of chromosomes (Cano et al., 1984), while the 20 largest scaffolds in our assembly are all larger than 14 Mb each, and contain together 85.4% of the total genome size. Contiguity is also high (contig N50 = 698.5 kb), which is comparable to other lacertids assembled using long-read sequencing (Supplementary Figure S3.1). The genome is highly complete, since a *BUSCO* search identified 93.7% of complete single-copy orthologues from a reference tetrapod database. The genome annotation identified 18,713 protein coding sequences, while a *BUSCO* search for tetrapod orthologues in the annotation indicated the presence of 78.3% complete single-copy, 0.9% complete duplicated and 8.9% fragmented orthologues.

Reduced genetic differentiation between high and low-altitude populations

To investigate selection associated with high-altitude adaptation in *G. galloti*, whole-genome pool-sequencing for four populations was performed. This yielded a total of 632,659,131 short-reads, which were aligned to the *G. galloti* reference genome assembly (Supplementary Table S3.2). After removal of PCR duplicates, an average of 14.6X coverage per pool was obtained, which mapped to the reference genome with high mapping rates (~99% of reads mapping).

Analyses started by examining the global patterns of genetic variation. Pairwise values of F_{ST} between the four populations, based on a total of 7,944,720 SNP (single nucleotide polymorphisms), showed moderate differentiation between populations, with average values ranging between 0.072 and 0.100 (Figure 3.2a). Importantly, no geographical pattern of stronger differentiation associated with altitude was recovered. Genetic variation (π and Tajima's D , Figure 3.2b and Figure 3.2c respectively) suggest a mixed pattern. π is similar across all populations, with median genome-wide values ranging between 0.69% (CT) and 0.66% (SA). Patterns of Tajima's D of each population present a common tendency towards negative values in all populations, albeit showing more variability between them. Additionally, the high-altitude population shows a

tendency to more negative Tajima's D which can be an effect of a recent population expansion after a bottleneck. Overall, these results indicate that populations of *G. galloti* inhabiting strikingly different altitudinal levels exhibit similar patterns of genetic variation.

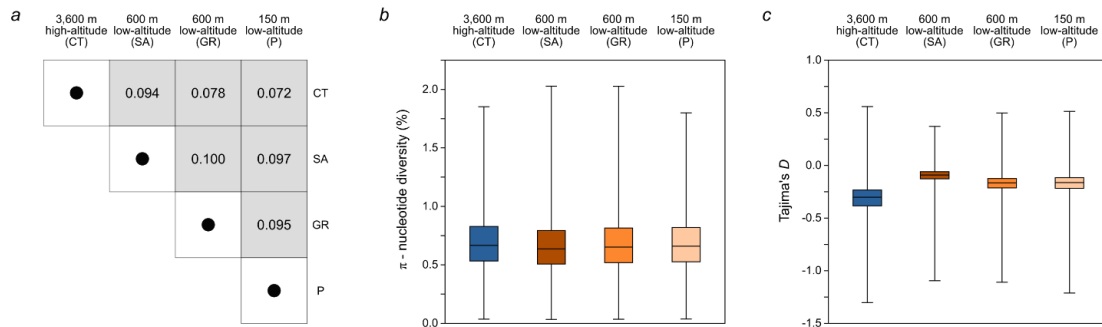


Figure 3.2. Population genomics of high and low-altitude *Gallotia galloti*. (a) Pairwise values of the fixation index (F_{ST}) between pools of samples from the four populations in the study. (b) Box-plots with distribution of genome-wide values of nucleotide diversity (π , %). (c) Box-plots distribution of genome-wide values of Tajima's D , with middle line representing the mean, box the 1st and 2nd quantiles and lines the 3rd and 4th quantiles. The three statistics were calculated for non-overlapping windows of 100 kb.

Relatively few signatures of selection for high-altitude adaptation

Next, using a sliding-window approach, little overall genetic differentiation across the genome between low and high-altitude populations was recovered (Figure 3.3a), as suggested in the previous analyses (see Figure 3.2). There was also a near-absence of signals that would suggest strong selection on specific loci in response to environmental constraints at high-altitude. As an exception, two genomic regions with moderate genomic differentiation were identified on scaffold 12 ($F_{ST} = 0.137$) and scaffold 17 ($F_{ST} = 0.175$). Two additional analyses were then performed in search of evidence of positive selection, namely variations in nucleotide diversity and allele frequency shifts associated with selection. These comparisons were polarized to highlight selective sweeps in high-altitude populations. Specifically, higher values of each statistic are indicative of genomic regions in which either π or Tajima's D values are lower in high-altitude populations, consistent with selective sweeps reducing local genetic diversity and leading to an excess of rare alleles. Results for both π -ratio (Figure 3.3b) and ΔTD (Figure 3.3c) are congruent with a scenario of overall reduced selection in high-altitude populations. Outlier regions were in general few and have little overlap with the relative outliers of the

F_{ST} scan, with the notable exceptions of the same two regions in scaffolds 12 (π -ratio = 1.944; $\Delta TD = 0.347$) and 17 (π -ratio = 1.858; $\Delta TD = 0.823$).

The three statistics (π and TD and F_{ST}) were in general uncorrelated: $\rho_{F_{ST}-\pi} = 0.174$; $\rho_{F_{ST}-TD} = 0.042$; $\rho_{\pi-TD} = 0.142$. The integration of these three statistics into DCMS data gave further support for a scenario of reduced overall selection, with few outliers relative to the genomic background level (Figure 3.3d). Considering the top 0.1% windows ($n = 91$) as the genomic regions with the best evidence of selection, these were grouped into 13 intervals (Table 3.1). As expected, the two intervals in scaffolds 12 (18 windows, 20% of the outliers) and 17 (31 windows, 34% of the outliers) were the two most striking outliers in this analysis.

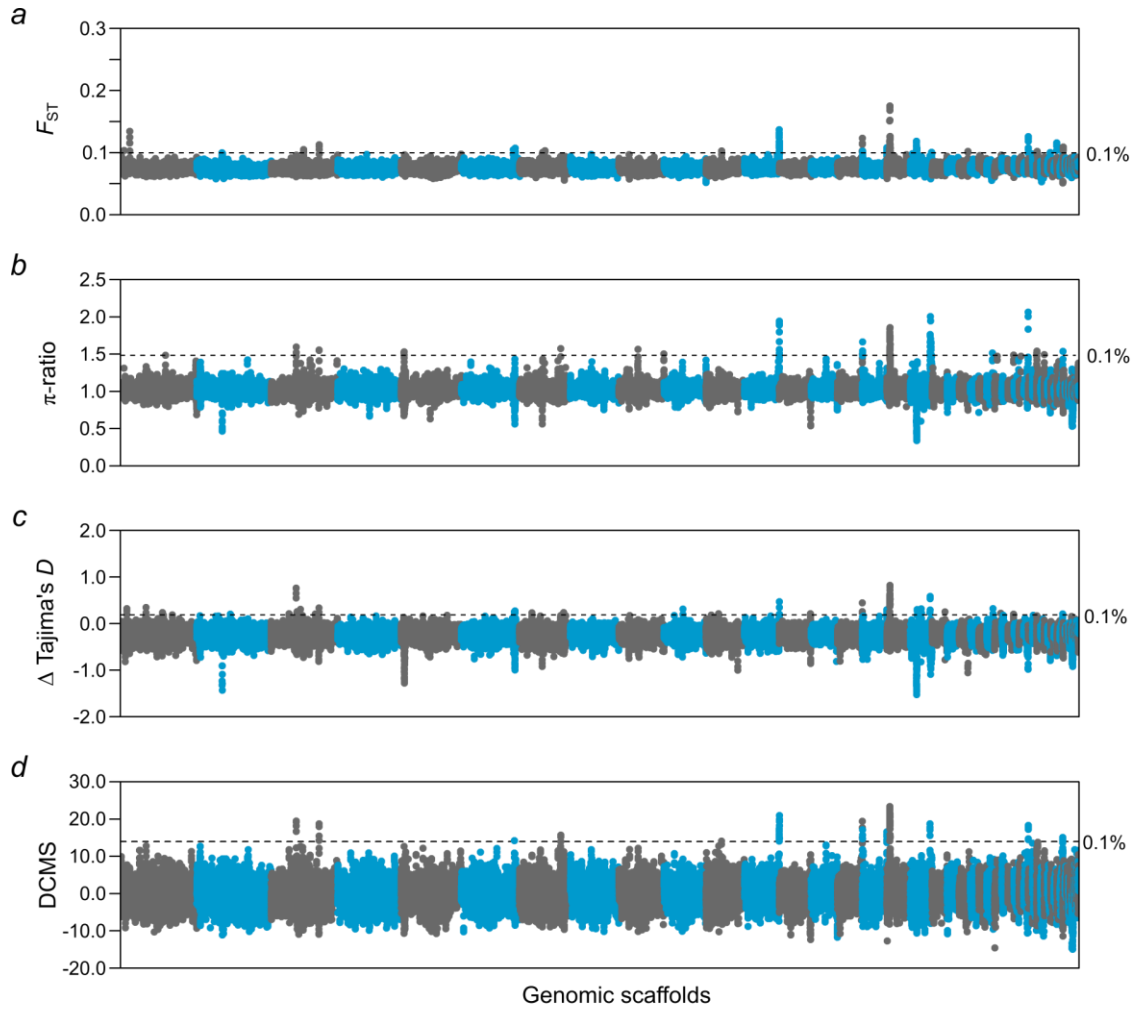


Figure 3.3. Genome-wide signatures of selection potentially associated with high-altitude adaptation in *Gallotia galloti*. (a) Fixation index (F_{ST}) between a high-altitude population and the combined data from three low-altitude populations. (b) Ratio between the nucleotide diversity (π , %) of low-altitude and high-altitude populations. (c) Differences in the allele frequency spectrum of low and high-altitude populations as calculated with Tajima's D . (d) De-correlated composite of multiple statistics (DCMS), calculated with values from the three statistics in panels a-c. The dashed line corresponds to the threshold indicating the top 0.1% windows in the distribution (windows above it were considered as representing strong evidence of selection). Each dot on the Manhattan plots represents one 50 kb window (windows were calculated with overlap of 12.5 kb). Colours represent alternating genomic scaffolds.

Table 3.1. Genomic intervals with evidence of selection to high-altitude in *Gallotia galloti*, highlighted from a composite selection statistic (DCMS) and ordered from highest to lowest DCMS value. Protein coding genes in each interval were retrieved from the *G. galloti* genome annotation, considering a 100 kb buffer either side of the genomic interval with evidence of selection, and were here given gene names from their human orthologues. Protein coding sequences with no known orthologues (marked with *) were given a specific code, following the annotation.

Scaffold number	Coordinate interval (kb)	Coordinate range (kb)	Genes within or overlapping an additional 100kb in each direction from the interval	Top DCMS value (50kb window)
17	3,425,000-4,062,500	637.5	<i>PPP2R2A</i> , <i>BNIP3L</i> , <i>DPYSL2</i> , <i>ADRA1A</i> , <i>Gal.00g130040*</i> , <i>Gal.00g130060*</i>	23.36
12	41,637,500-41,895,811	258.3	<i>BMPER</i> , <i>FANCD2OS</i> , <i>NPSR1</i>	21
3	29,875,000-29,962,500	87.5	<i>RPS6KA3</i> , <i>CNKSR2</i> , <i>BCOR</i>	19.48
15	29,625,000-29,712,500	87.5	<i>TLN2</i>	19.4
3	57,362,500-57,450,000	87.5		18.72
18	23,575,000-23,675,000	100	<i>DNM1</i> , <i>CIZ1</i> , <i>BBLN</i> , <i>PTGES2</i> , <i>ODF2</i> , <i>ABCA2</i> , <i>FUT7</i> , <i>NPDC1</i>	18.72
30	5,437,500-5,533,213	95.7	<i>TPBG</i> , <i>Gal.00g165360*</i> , <i>C7orf25</i>	18.31
16	362,500-437,500	75	<i>SLC25A3</i> , <i>DMRTB1</i> , <i>GLIS1</i> , <i>NDC1</i>	17.13
16	29,287,500-29,450,000	162.5	<i>ASTN1</i> , <i>PAPP2</i> , <i>PEG10</i> , <i>TECR</i>	16.56
7	49,537,500-49,612,500	75	<i>RERE</i> , <i>ENO1</i> , <i>CA6</i> , <i>SLC2A5</i>	15.72
40	3,350,000-3,462,500	112.5	<i>COP1</i>	15.11
6	63,725,000-63,775,000	50	<i>SPG16</i>	14.21
11	18,437,500-18,487,500	50	<i>LRIG2</i> , <i>MAGI3</i>	14.09

Signatures of selection overlap candidate genes for high-altitude adaptation

To check for putative signatures of selection in the 13 regions with windows that exceeded the threshold in the DCMS analysis, we inspected the genome annotation for the presence of protein coding genes and coding mutations with potential functional implications (Table 3.1). For the top outlier region (scaffold 17:3,425,000-4,062,500), the highest values of F_{ST} and lower values of π and Tajima's D in the high-altitude populations were coincident, and overlapped the open reading frame of the gene coding for dihydropyrimidinase-like 2 (*DPYSL2*, Figure 3.4, Table 3.1). To check for the presence of protein coding changes, all the exons of the gene were inspected, but only three SNP found that coded for synonymous changes and that had a low-to-moderate allele frequency difference between low and high-altitude populations ($\Delta AF = 0.034$ - 0.214), plus an additional splice site variant with low allele frequency differences between the two contrasts ($\Delta AF = 0.035$). This suggests that selection in this region is targeting regulatory variants. To explore additional genes that could be potential targets of selection on regulatory variants in this region, search was extended to 100 kb either side of the interval mentioned above (this should include the majority of long-range regulatory interactions, Chen et al., 2020). A large number of intronic and intergenic SNP with high allele frequency differentiation in this broader interval ($\Delta AF \geq 0.5$, $n = 92$) were found, encompassing the orthologues for the genes *DPYSL2*, *BNIP3L*, *ADRA1A* and *PPP2R2A*, plus two annotated protein-coding sequences for which we could not confirm their homology. For these two protein-coding sequences four missense variants with moderate allele frequency differences ($\Delta AF = 0.319$ - 0.344) were found, but given the lack of clear homology of these sequences, they may correspond to annotation artefacts.

Considering described protein functions in other vertebrates, some of these genes are good candidates to mediate high-altitude adaptation in *G. galloti*, in particular those associated with functions such as hypoxia-induced mitochondrial autophagy (*BNIP3L*, Bellot et al., 2009; see also review in Murray, 2009) or response to hypoxia-induced pulmonary vasoconstriction (*ADRA1A*, Moretta et al., 2019). An absence of called variants within a approx. 93 kb region near these genes (scaffold 17: 3,676,883-3,776,884) coincides with a gap in our reference assembly. In the genome assembly of *Podarcis muralis*, another lacertid with a highly complete annotation (Ensembl Release 106, April 2022; Supplementary Figure S3.1), the homologous region contains only two protein coding sequences, a homologue of a zinc-finger protein (*XICOF6*-like) with uncertain functions, and a reverse transcriptase.

The second highest outlier region was located at the terminal end of scaffold 12 (scaffold 12:41,637,500-41,895,811), and is likely to be in synteny with the outlier region at the terminal end of scaffold 40 (scaffold 40:3350000-3462500), according to the *Podarcis muralis* assembly. These regions contain the genes *BMPER*, *FANCD2OS*, *NSPR1* (scaffold 12) and *COP1* (scaffold 40), with no other genes within a 100 kb range (Figure 3.4, Table 3.1). The signal of genetic differentiation between low and high-altitude pools was particularly elevated within the open reading frame of *BMPER*, which harboured the three missense variants with moderate allele frequency differences in this region ($\Delta AF = 0.102-0.215$). A further 15 highly differentiated intronic and intergenic variants were found across the outlier region in scaffold 12, and four highly differentiated variants in scaffold 40. *BMPER* is a regulator of bone morphogenetic proteins (BMPs) in endothelial tissues, fine-tuning the activity of BMPs in angiogenesis (Heinke et al., 2008). This link makes *BMPER* an excellent candidate for selection in response to chronic hypoxia through pulmonary vascular remodelling (Frank et al., 2005).

In the remaining 11 outlier regions we found a further 30 missense variants, of which 15 had moderate allele frequency differences ($\Delta AF = 0.100-0.279$). The number of highly differentiated variants ranged from 3 to 17 per region, suggesting that both regulatory and coding variation play a role in adaptation. Orthologues of many of the genes within these 11 candidate intervals (or paralogs of it) have been previously referenced to be up or down-regulated in hypoxic conditions (iHypoxia database, Liu et al., 2021), for example, through hypoxia-inducible transcription factors (Kaelin & Ratcliffe 2008).

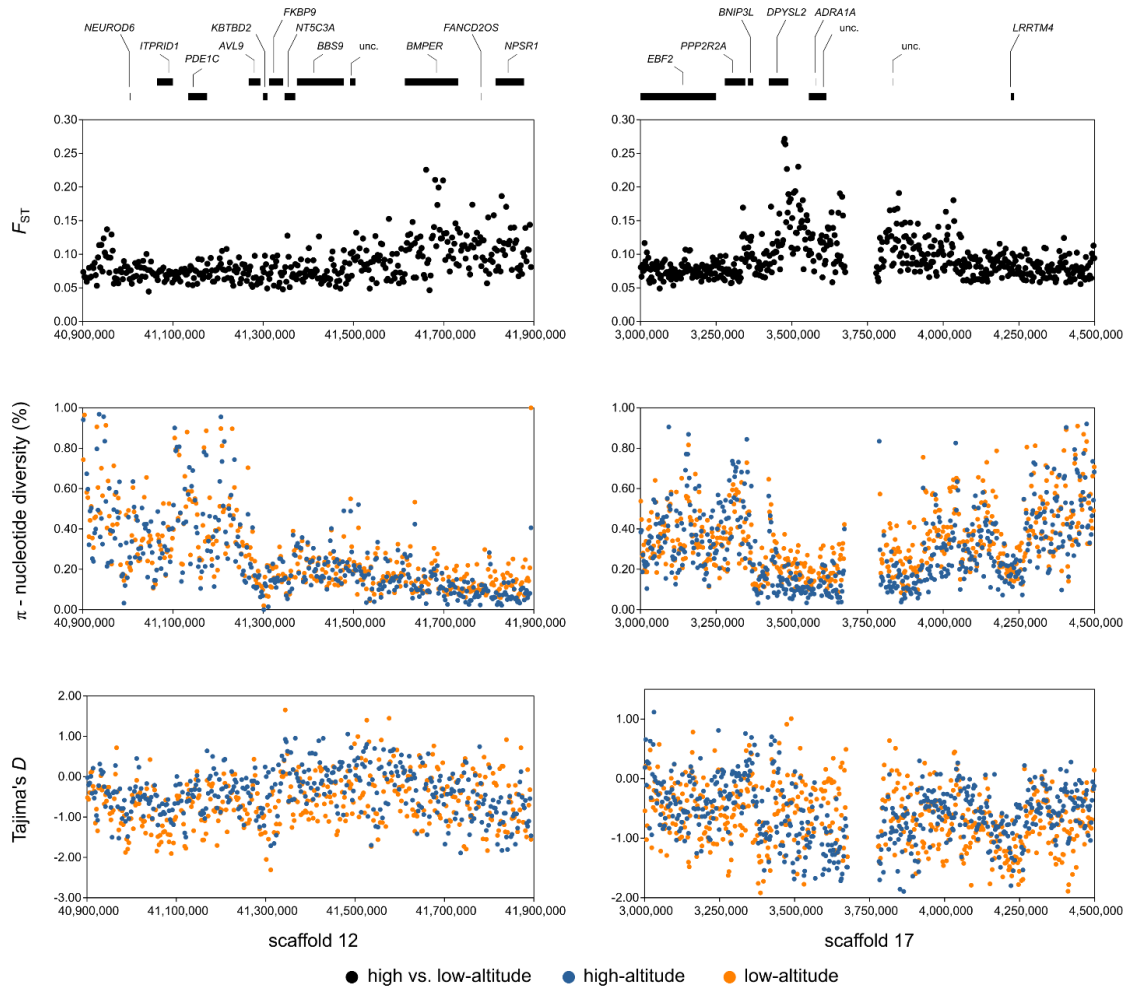


Figure 3.4. Localized genomic signatures of adaptation to altitude in *Gallotia galloti*. Detailed view of patterns of genetic differentiation (F_{ST}), variation (π , %) and allele frequency spectrum (Tajima's D) for the two most important outlier regions in our genome scans. Each dot represents a non-overlapping 2.5 kb window (results calculated using the same parameters as for the genomic scan). Annotated genes in these regions are shown on top (gene names adapted from the human orthologues).

3.5. Discussion

Extensive research has been conducted to understand how organisms cope with high altitude environments. However, there is still an important lack of knowledge on the genetic mechanisms that mediate altitudinal adaptation in ectotherms. In this study, we analysed signatures of selection in an outstanding lizard model that occupies a continuous altitudinal range from sea-level to the summit of a volcano at 3,700 meters. Our analyses suggest little differentiation between populations living at low and high altitude, and the presence of a few and highly localized genetic signatures of adaptation to high altitude. We examined the patterns of genetic variation in these regions in detail, and showed that the two most striking outliers in a composite selection scan are located near genes that have been associated with response to hypoxia in medical studies.

Hypoxic conditions are amongst the most physiologically-demanding natural-occurring phenomena in high elevation habitats (reviewed in Pamenter et al., 2020). The fact that in our high-altitude population, the two strongest selection signatures are associated with genes involved in different responses to hypoxia is further evidence of the essential role that these functions play in the colonization and survival of organisms at high altitudes, as in the case of our study species, *G. galloti*. This is no surprise: above 3,000 metres, atmospheric oxygen pressure drops by more than 30% compared to normal pressure at sea-level (Chavala, 2018). When exposed to reduced O₂ availability, animals from low altitudes alter their phenotype by increasing pulmonary ventilation, hemoglobin concentration, and heart rate (Banchero, 1987; reviewed in Storz & Scott, 2019). However, although plastic adaptation responses can immediately counteract hypoxia, prolonged hypoxic conditions (chronic hypoxia) are associated with serious pathological conditions that are often driven by some of these plastic responses themselves, such as high-altitude pulmonary-edema and pulmonary hypertension (Storz & Scott, 2019). *BMPER* and *ADRA1A*, two regulators of vascular cell growth and proliferation, are thus excellent candidates to mediate adaptation to evade chronic hypoxia in *G. galloti* populations living at high-altitudes. *BMPER* is an endothelial regulator of bone morphogenetic proteins, which in turn are essential tissue “architects”. In mammals, *BMPER* (BMP-binding endothelial regulator) is expressed in skin, heart and lung tissue, where it is essential for *BMP4*-mediated angiogenesis (formation of new blood vessels; Heinke et al., 2008), whereas *BMP4* has been shown to be upregulated in lung tissue during hypoxia to promote proliferation and remodelling of arterial smooth muscle (Frank et al., 2005; Wu et al., 2014). *ADRA1A*, Adrenoceptor alpha 1A, is an

adrenergic receptor that binds to epinephrine and norepinephrine in cells of various tissues and contributes to the response to cardiac pressure overload (O'Connell et al., 2006). *ADRA1A* has been shown to be downregulated under hypoxia in snapping turtle embryos (Eme et al., 2013) and upregulated in embryonic and adult sheep (Moretta et al., 2019), so more studies would be necessary to clarify the role of this gene in mitigating hypoxia.

Near *ADRA1A* (~184 kb) is *BNIP3L* (BCL2-interacting protein 3-like), which is thought to contribute to high-altitude adaptation. *BNIP3L* encodes a protein that belongs to a pro-apoptotic subfamily of proteins that promote mitochondrial autophagy (Li et al., 2021). Crucially, *BNIP3L* expression can be induced by hypoxia-inducible factor-1alpha (*HIF1A*), meaning that *BNIP3L*-mediated mitochondrial autophagy is promoted under hypoxic conditions (Bellot et al., 2009; Marinković & Novak 2021). Reductions in mitochondrial volume density with altitude have been observed both in acclimatised human climbers and adapted highland populations (Murray & Horscroft, 2016), supporting the idea that genes controlling for mitochondrial autophagy should be likely targets of selection, although selection on mitochondrial function is also dependent on life-history traits (Gangloff et al., 2020).

Although both *ADRA1A* and *BNIP3L* were located in close proximity to the major outlier region in our genome scan and both seem good candidates for adaptation in our system, it should not be ignored that the majority of the highly differentiated SNP overlaps with another gene, *DPYSL2*. This gene is associated with neuron development, and mutations in this gene lead to neurological diseases (Ip et al., 2014). In turtles, oxygen deprivation in the brain is known to have an immediate effect, triggering rapid cellular responses that reduce metabolic demand (Lutz & Prentice, 2002). A recent study (Brustovetsky et al., 2021) suggests an additional role in regulating mitochondrial morphology and motility in neuronal cells, but the exact (if any) adaptive role of this mechanism is still unknown. Other genes within our candidate regions, such as *ENO1* (associated with resistance to hypoxia in pancreatic cancer cells; Wang et al., 2019) or *TPBG* (regulates angiogenic activity of pericytes; Spencer et al., 2019) may have additional roles, but without further detailed physiological testing and functional genomic studies, their roles remain unclear.

Overall, our study provides crucial evidence for an ectotherm that may generalise previous findings in endotherms that hypoxia acts as the most demanding selection pressure in high-altitude environments. However, there are a variety of other abiotic stressors in these environments, such as UV, temperature and water availability

differences, that likely contribute to selection processes but in our study no clear signatures of selection for such other stressors have been detected. For example, increased UV exposure should (in principle) drive selection for a noticeable increased pigmentation, but we did not find melanin-pathway genes in our outliers. This may occur because lizards actively regulate for UV exposure, something that has been recently recognized (Conley & Lattancio, 2022).

It is therefore possible that high-altitude populations of *G. galloti* are able to respond to these challenges through phenotypic plasticity (e.g. in morphology, shifts in growth), behavioural changes (behavioural thermoregulation and UV-regulation), physiological mechanisms (hydroregulatory mechanisms, metabolic rates), or other mechanisms (Bels & Russel, 2019; Muñoz, 2021). This is also consistent with our main conclusions from the ecophysiology chapter of the current thesis, where we found no striking differences between populations at different altitudes (see Chapter II). Genetic adaptation and phenotypic plasticity thus likely play complex and interdependent roles in mediating responses to high abiotic stress in ectotherms.

3.6. Acknowledgements

This work was funded by the project GCRESPONSE – POCI-01-0145-FEDER-028014. N.S. was supported by a research fellowship SFRH/BD/128612/2017 and extension grant (COVID/BD/152580/2022); P.A. was supported by FCT through research contract 2020.01405.CEECIND/CP1601/CT0011. C.P. was supported by Portuguese national funds through FCT-Fundação para a Ciência e a Tecnologia in the scope of the project UIDB/50027/2020. A.Ž. was funded from the state budget by the Slovenian Research Agency (Programme P1-0255 and grant J1-2466). We thank to Manuel Nogales, Alicia Boto, Marta López-Darias IPNA-CSIC, Miguel Molina-Borja (Universidad de La Laguna) and Beatriz Fariña (grupo Perequén AHE) for providing the necessary conditions and support for the research in Tenerife at Instituto de Productos Naturales y Agrobiología, IPNA, CSIC and Miha Krofel for help in the sampling campaigns. Sampling was allowed under permits AFF 57/17 2017-00308, exit record: 13413, AFF 67/17 2017-00335, exit record: 15807, AFF 160/18 2018-02258, exit record: 28466; and Teide National Park sampling permit JLRG/arm, exit record 138/2 issued by Cabildo Insular de Tenerife.

3.7. Data availability

Raw 10X Genomics Chromium sequencing data and whole-genome pool-sequencing data are available in the Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA833592.

3.8. References

Ancochea, E., Fuster, J., Ibarrola, E., Cendrero, A., Coello, J., Hernan, F., Cantagrel, M. & Jamond, C. (1990). Volcanic evolution of the island of Tenerife (Canary Islands) in the light of new K-Ar data. *Journal of Volcanology and Geothermal Research*, 44(3-4), 231-249.

Andrade, P., Pinho, C., i de Lanuza, G. P., Afonso, S., Brejcha, J., Rubin, C. J., Wallerman, O., Pereira, P., Sabatino, S. J., Bellati, A., Pellitteri-Rosa, D., Bosakova, Z., Bunikis, I., Carretero, M. A., Feiner, N., Marsik, P., Paupéio, F., Salvi, D., Soler, L., While, G. M., Uller, T., Font, E., Andersson & Carneiro, M. (2019). Regulatory changes in pterin and carotenoid genes underlie balanced color polymorphisms in the wall lizard. *Proceedings of the National Academy of Sciences*, 116(12), 5633-5642.

Angilletta MJ (2009). Thermal adaptation: a theoretical and empirical synthesis. USA Oxford University Press, ISBN 978-0198570882.

Banchero, N. (1987). Cardiovascular responses to chronic hypoxia. *Annual review of physiology*, 49(1), 465-476.

Bellot, G., Garcia-Medina, R., Gounon, P., Chiche, J., Roux, D., Pouysségur, J., & Mazure, N. M. (2009). Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Molecular and cellular biology*, 29(10), 2570-2581.

Bels, V. & Russell, A. (2019). eds. *Behavior of lizards: evolutionary and mechanistic perspectives*. Crc Press.

Blumthaler, M., Ambach, W., & Ellinger, R. (1997). Increase in solar UV radiation with altitude. *Journal of photochemistry and Photobiology B: Biology*, 39(2), 130-134.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114-2120.

Bradnam, K. R., Fass, J. N., Alexandrov, A., Baranay, P., Bechner, M., Birol, I., Boisvert, S., Chapman, J. A., Chapuis, G., Chikhi, R., Chitsaz, H., Chou, W.-C., Corbeil, J., Del Fabbro, C., Docking, T. R., Durbin, R., Earl, D., Emrich, S., Fedotov, P., Fonseca, N. A., et al. (2013). Assemblathon 2: evaluating de novo methods of genome assembly in three vertebrate species. *GigaScience*, 2(1), 2047-217X.

- Brown, R. P., Paterson, S., & Risse, J. (2016). Genomic signatures of historical allopatry and ecological divergence in an island lizard. *Genome Biology and Evolution*, 8(11), 3618-3626.
- Brustovetsky, T., Khanna, R., & Brustovetsky, N. (2021). Crmp2 is involved in regulation of mitochondrial morphology and motility in neurons. *Cells*, 10(10), 2781.
- Cano, J., Baez, M., López-Jurado, L. F., & Ortega, G. (1984). Karyotype and chromosome structure in the lizard, *Gallotia galloti* in the Canary Islands. *Journal of Herpetology*, 18(3), 344-346.
- Carracedo, J. C., Badiola, E. R., Guillou, H., Paterne, M., Scaillet, S., Torrado, F. Paris, R., Fra-Paleo, U. and Hansen, A. (2007). Eruptive and structural history of Teide Volcano and rift zones of Tenerife, Canary Islands. *Geological Society of America Bulletin*, 119(9-10), 1027-1051.
- Chavala, M. A. (2018). A journey between high altitude hypoxia and critical patient hypoxia: What can it teach us about compression and the management of critical disease?. *Medicina Intensiva (English Edition)*, 42(6), 380-390.
- Chen, C. H., Zheng, R., Tokheim, C., Dong, X., Fan, J., Wan, C., Tang, Q., Brown, M., Liu, J. S., Meyer, C. A. & Liu, X. S. (2020). Determinants of transcription factor regulatory range. *Nature communications*, 11(1), 1-15.
- Cheviron, Z. A., Whitehead, A., & Brumfield, R. T. (2008). Transcriptomic variation and plasticity in rufous - collared sparrows (*Zonotrichia capensis*) along an altitudinal gradient. *Molecular ecology*, 17(20), 4556-4569
- Chiou, K. L., Janiak, M. C., Schneider-Crease, I. A., Sen, S., Ayele, F., Chuma, I. S., Knauf, S., Lemma, A., Signore, A. V., D'Ippolito, A. M., Abebe, B., Haile, A. A., Kebede F., Fashing, P. J., Nguyen, N., McCann, C., Houck, M. L., Wall, J. D., Burrell, A.S., Bergey, C. M., et al. & Snyder-Mackler, N. (2022). High-altitude adaptation and incipient speciation in geladas. *Nature ecology & evolution*, 6(5), 630-643
- Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., Land, S. J., Lu, X., & Ruden, D. M. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly*, 6(2), 80-92.
- Conley, D. A., & Lattanzio, M. S. (2022). Active regulation of ultraviolet light exposure overrides thermal preference behaviour in eastern fence lizards. *Functional Ecology*, 36(9), 2240-2250.

- Cox, S. C., Carranza, S., & Brown, R. P. (2010). Divergence times and colonization of the Canary Islands by *Gallotia* lizards. *Molecular Phylogenetics and Evolution*, 56(2), 747-757.
- Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., Whitwham, A., Keane, T., McCarthy, S. A., Davies, R. M. & Li, H. (2021). Twelve years of SAMtools and BCFtools. *Gigascience*, 10(2), giab008
- Eme, J., Rhen, T., Tate, K. B., Gruchalla, K., Kohl, Z. F., Slay, C. E., & Crossley, D. A. (2013). Plasticity of cardiovascular function in snapping turtle embryos (*Chelydra serpentina*): chronic hypoxia alters autonomic regulation and gene expression. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 304(11), R966-R979.
- Fan, Z., Ortega-Del Vecchyo, D., & Wayne, R. K. (2017). EPAS1 variants in high altitude Tibetan wolves were selectively introgressed into highland dogs. *PeerJ*, 5, e3522.
- Fariña, B., Rodríguez, M. L., López, M. R. and Moreno, J. E. 2011. Sobre la presencia de *Gallotia galloti* en el pico del volcán Teide (Tenerife, Islas Canarias). *Boletín de la Asociación Herpetológica Española*, 22, 46-51
- Fernández-Rodríguez, I.; Barroso, F. M. & Carretero, M. A. (2021). An integrative analysis of the short-term effects of tail autotomy on thermoregulation and dehydration rates in wall lizards (*Podarcis bocagei*). *Journal of Thermal Biology*, 99, 102976.
- Flynn, J. M., Hubley, R., Goubert, C., Rosen, J., Clark, A. G., Feschotte, C., & Smit, A. F. (2020). RepeatModeler2 for automated genomic discovery of transposable element families. *Proceedings of the National Academy of Sciences*, 117(17), 9451-9457
- Frank, D. B., Abtahi, A., Yamaguchi, D. J., Manning, S., Shyr, Y., Pozzi, A., Baldwin, H. S., Johnson, J. E., & De Caestecker, M. P. (2005). Bone morphogenetic protein 4 promotes pulmonary vascular remodeling in hypoxic pulmonary hypertension. *Circulation research*, 97(5), 496-504.
- Gangloff, E. J., Schwartz, T. A., Klabacka, R., Huebschman, N., Lui, A. Y. and Bronikowski, A. M. 2020. Mitochondria as central characters in a complex narrative: Linking genomics, energetics, pace-of-life, and aging in natural populations of garter snakes. - *Experimental Gerontology* 137
- García-Muñoz, E.; Ceacero, F., Pedrajas, L., Kaliontzopoulou, A., & Carretero, M. A. (2011): Tail tip removal for tissue sampling has no short-term effects on microhabitat

selection by *Podarcis bocagei*, but induced autotomy does. *Acta Herpetologica*, 6(2): 223-227

Gazda, M. A., Andrade, P., Afonso, S., Dilyt , J., Archer, J. P., Lopes, R. J., Faria, R. & Carneiro, M. (2018). Signatures of selection on standing genetic variation underlie athletic and navigational performance in racing pigeons. *Molecular Biology and Evolution*, 35(5), 1176-1189.

Gonz lez-Morales, J. C., Rivera-Rea, J., Moreno-Rueda, G., Bastiaans, E., D  az-Albiter, H., D  az de la Vega-P  rez, A. H., Bautista, A., & Fajardo, V. (2021). To be small and dark is advantageous for gaining heat in mezquite lizards, *Sceloporus grammicus* (Squamata: Phrynosomatidae). *Biological Journal of the Linnean Society*, 132(1), 93-103.

Gou, X., Wang, Z., Li, N., Qiu, F., Xu, Z., Yan, D., Yang, S., Jia, J., Kong, X., Wei, Z., Lu, S., Lian, L., Wu, C., Wang, X., Li, G., Ma, T., Jiang, Q., Zhao, X., Yang, J., et al., & Li, Y. (2014). Whole-genome sequencing of six dog breeds from continuous altitudes reveals adaptation to high-altitude hypoxia. *Genome research*, 24(8), 1308-1315.

Haas, B. J., Salzberg, S. L., Zhu, W., Pertea, M., Allen, J. E., Orvis, J., White, O., Buell, R. & Wortman, J. R. (2008). Automated eukaryotic gene structure annotation using EvidenceModeler and the Program to Assemble Spliced Alignments. *Genome biology*, 9(1), 1-22.

Halbritter, A. H., Fior, S., Keller, I., Billeter, R., Edwards, P., Holderegger, R., et al. (2018). Trait differentiation and adaptation of plants along elevation gradients. *Journal of Evolutionary Biology*, 31, 784–800. doi: 10.1111/jeb.13262.

Heinke, J., Wehofsits, L., Zhou, Q., Zoeller, C., Baar, K. M., Helbing, T., Laib, A., Augustin, H., Bode, C., Patterson, C. & Moser, M. (2008). BMPER is an endothelial cell regulator and controls bone morphogenetic protein-4–dependent angiogenesis. *Circulation research*, 103(8), 804-812.

Hendrickson, S. L. (2013). A genome wide study of genetic adaptation to high altitude in feral Andean Horses of the p  ramo. *BMC evolutionary biology*, 13(1), 1-13.

Howald, H., & Hoppeler, H. (2003). Performing at extreme altitude: muscle cellular and subcellular adaptations. *European journal of applied physiology*, 90(3), 360-364.

Huerta-S  nchez, E., Jin, X., Bianba, Z., Peter, B. M., Vinckenbosch, N., Liang, Y., Yi, X., He, M., Somel, M., Ni, P., Wang, B., Ou, X., H., Luosang, J., Xi, Z., Cuo, P., Li, K., Gao, G., Yin, Y., Wang, W., Zhang, X., Xu, X., Yang, H. & Nielsen, R. (2014). Altitude

adaptation in Tibetans caused by introgression of Denisovan-like DNA. *Nature*, 512(7513), 194-197.

Humann, J. L., Lee, T., Ficklin, S., & Main, D. (2019). Structural and functional annotation of eukaryotic genomes with GenSAS. In *Gene prediction* (pp. 29-51). Humana, New York, NY

Ip, J. P., Fu, A. K., & Ip, N. Y. (2014). CRMP2: functional roles in neural development and therapeutic potential in neurological diseases. *The Neuroscientist*, 20(6), 589-598.

Jiang, Z. W., Ma, L., Mi, C. R., & Du, W. G. (2021). Effects of hypoxia on the thermal physiology of a high-elevation lizard: implications for upslope-shifting species. *Biology Letters*, 17(3), 20200873.

Jørgensen, S.E, Fath, B. D. (2008). *Encyclopedia of Ecology* - <https://www.sciencedirect.com/referencework/9780080454054/encyclopedia-of-ecology> ISBN 978-0-08-045405-4, Elsevier.

Kaelin Jr, W. G., & Ratcliffe, P. J. (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Molecular cell*, 30(4), 393-402.

Kapun, M., Barrón, M. G., Staubach, F., Obbard, D. J., Wiberg, R. A. W., Vieira, J., Goubert, C., Rota-Stabelli, O., Kankare, M., Bogaerts-Márquez, M., Haudry, A., Waidele, L., Kozeretska, I., Pasyukova, E. G., Loeschcke, V., Pascual, M., Vieira, C. P., Serga, I., Monthchamp-Moreau, C. & González, J. (2020). Genomic analysis of European *Drosophila melanogaster* populations reveals longitudinal structure, continent-wide selection, and previously unknown DNA viruses. *Molecular Biology and Evolution*, 37(9), 2661-2678.

Kofler, R., Orozco-terWengel, P., De Maio, N., Pandey, R. V., Nolte, V., Futschik, A., Kosiol, C & Schlötterer, C. (2011b). PoPoolation: a toolbox for population genetic analysis of next generation sequencing data from pooled individuals. *PloS one*, 6(1), e15925.

Kofler, R., Pandey, R. V., & Schlötterer, C. (2011a). PoPoolation2: identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq). *Bioinformatics*, 27(24), 3435-3436.

Kolora, S. R. R., Weigert, A., Saffari, A., Kehr, S., Walter Costa, M. B., Spröer, C., Indrischek, H., Chintalapati, M., Lohse, K., Doose, G., Overmann, J., Bunk, B., Bleidorn, C., Grimm-Seyfarth, A., Henle, K., Nowick, K., Faria, R., Stadler, P. F., & Schlegel, M.

- (2019). Divergent evolution in the genomes of closely related lacertids, *Lacerta viridis* and *L. bilineata*, and implications for speciation. *Gigascience*, 8(2), giy160.
- Li, H. (2013) Aligning sequence reads, clone sequences and assembly contigs with BWAMEM. (2013). *ArXiv*:1303.3997.
- Li, J. T., Gao, Y. D., Xie, L., Deng, C., Shi, P., Guan, M. L., Huang, S., Ren, J. L., Wu, D. D., Ding, L., Huang, Z. Y., Nie, H., Humphreys, D. P., Hillis, D. M., Wang, W. Z. & Zhang, Y. P. (2018). Comparative genomic investigation of high-elevation adaptation in ectothermic snakes. *Proceedings of the National Academy of Sciences*, 115(33), 8406-8411.
- Li, Y., Zheng, W., Lu, Y., Zheng, Y., Pan, L., Wu, X., Yuan, Y., Shen, Z., Ma, S., Zhang, X., Wu, J., Chen, Z., & Zhang, X. (2021). BNIP3L/NIX-mediated mitophagy: molecular mechanisms and implications for human disease. *Cell death & disease*, 13(1), 1-11.
- Liu, X., Zhang, Y., Li, Y., Pan, J., Wang, D., Chen, W., Zheng, Z., He, X., Zhao, Q., Pu, Y., Guan, W., Han, J., Orlando, L., Ma, W. & Jiang, L. (2019). EPAS1 gain-of-function mutation contributes to high-altitude adaptation in Tibetan horses. *Molecular biology and evolution*, 36(11), 2591-2603.
- Liu, Z. X., Wang, P., Zhang, Q., Li, S., Zhang, Y., Guo, Y., Jia, C., Shao, T., Li, L., Cheng, H. & Wang, Z. (2021). iHypoxia: an integrative database of the expression dynamics of proteins in response to hypoxia in animals. *bioRxiv*.
- Lutz, P. L., & Prentice, H. M. (2002). Sensing and responding to hypoxia, molecular and physiological mechanisms. *Integrative and Comparative Biology*, 42(3), 463-468.
- Ma Y, Ding X, Qanbari S, Weigend S, Zhang Q, Simianer H. (2015). Properties of different selection signature statistics and a new strategy for combining them. *Heredity* 115(5):426–436.
- Malik, A., Korol, A., Weber, M., Hankeln, T., Avivi, A., & Band, M. (2012). Transcriptome analysis of the spalax hypoxia survival response includes suppression of apoptosis and tight control of angiogenesis. *BMC genomics*, 13(1), 1-20.
- Marinković, M., & Novak, I. (2021). A brief overview of BNIP3L/NIX receptor-mediated mitophagy. *FEBS Open bio*, 11(12), 3230-3236.
- Monge, C., & F., Léon-Velarde. (1991). Physiological adaptation to high altitude: oxygen transport in mammals and birds. *Physiological reviews*, 71(4), 1135-1172.

Moretta, D., Papamatheakis, D. G., Morris, D. P., Giri, P. C., Blood, Q., Murray, S., Ramzy, M., Romero, M., Vemulakonda, S., Lauw, S., Longo, L. D., Zhang, L. & Wilson, S. M. (2019). Long-term high-altitude hypoxia and alpha adrenoceptor-dependent pulmonary arterial contractions in fetal and adult sheep. *Frontiers in Physiology*, 1032.

Muñoz, M. M. (2021). The Bogert effect, a factor in evolution. *Evolution*, 76, 49-66.

Murray, A. J. (2009). Metabolic adaptation of skeletal muscle to high altitude hypoxia: how new technologies could resolve the controversies. *Genome medicine*, 1(12), 1-9.

Murray, A. J., & Horscroft, J. A. (2016). Mitochondrial function at extreme high altitude. *The Journal of physiology*, 594(5), 1137-1149.

Navas, C. A. (1997). Thermal extremes at high elevations in the Andes: physiological ecology of frogs. *Journal of thermal biology*, 22(6), 467-477.

Nei, M. (1987). *Molecular evolutionary genetics*. Columbia university press.

O'Connell, T. D., Swigart, P. M., Rodrigo, M. C., Ishizaka, S., Joho, S., Turnbull, L., Tecott, L. H., Baker, A. J., Foster, E., Grossman, W. & Simpson, P. C. (2006). α 1-Adrenergic receptors prevent a maladaptive cardiac response to pressure overload. *The Journal of clinical investigation*, 116(4), 1005-1015.

Osojnik, N., Žagar, A., Carretero, M.A., García-Muñoz, E., Vrezec, A. (2013). Ecophysiological dissimilarities of two sympatric lizards. *Herpetologica*, 69, 445-454.

Paaïjmans, K. P., Heinig, R. L., Seliga, R. A., Blanford, J. I., Blanford, S., Murdock, C. C., & Thomas, M. B. (2013). Temperature variation makes ectotherms more sensitive to climate change. *Global change biology*, 19(8), 2373-2380.

Pamenter, M. E., Hall, J. E., Tanabe, Y., & Simonson, T. S. (2020). Cross-species insights into genomic adaptations to hypoxia. *Frontiers in genetics*, 11, 743.

Qu, Y., Zhao, H., Han, N., Zhou, G., Song, G., Gao, B., Tian, S., Zhang, J., Zhang, R., Meng, X., Zhang, Y., Zhang, Y., Zhu, X., Wang, W., Lambert, D., Ericson, P. G. P., Subramanian, S., Yeung Q., Zhu, C. H., et al. & Lei, F. (2013). Ground tit genome reveals avian adaptation to living at high altitudes in the Tibetan plateau. *Nature communications*, 4(1), 1-9.

Quagliariello, A., Di Paola, M., De Fanti, S., Gneccchi-Ruscione, G. A., Martinez-Priego, L., Pérez-Villaroya, D., Sherpa, M. G., Sherpa, P. T., Marinelli, G., Natali, L., Di Marcello, M., Peluzzi, D., Di Cosimo, P. D'Auria, G., Pettener, G., Sazzini, M., Luiselli D. & De Filippo, C. (2019). Gut microbiota composition in Himalayan and Andean populations

and its relationship with diet, lifestyle and adaptation to the high-altitude environment. *Journal of Anthropological Science*, 96, 189-208.

Reguera, S., Zamora-Camacho, F. J., & Moreno-Rueda, G. (2014). The lizard *Psammodromus algirus* (Squamata: Lacertidae) is darker at high altitudes. *Biological Journal of the Linnean Society*, 112(1), 132-141.

Richard, M., & Thorpe, R. S. (2001). Can microsatellites be used to infer phylogenies? Evidence from population affinities of the Western Canary Island lizard (*Gallotia galloti*). *Molecular Phylogenetics and Evolution*, 20(3), 351-360.

S'khifa, A., Carretero, M. A., Harris, D. J., & Slimani, T. (2022). Ecophysiological conservativeness and size-mediated plasticity in the High Mountain Lizard *Atlantolacerta andreanskyi* confirm its vulnerability to climate change. *Salamandra*, 58(2), 139-150.

Sannolo, M., Civantos, E., Martín, J., & Carretero, M. A. (2020). Variation in field body temperature and total evaporative water loss along an environmental gradient in a diurnal ectotherm. *Journal of Zoology*, 310(3), 221-231.

Schweizer, R. M., Velotta, J. P., Ivy, C. M., Jones, M. R., Muir, S. M., Bradburd, G. S., Storz, J. F., Scott, G. R. & Cheviron, Z. A. (2019). Physiological and genomic evidence that selection on the transcription factor *Epas1* has altered cardiovascular function in high-altitude deer mice. *PLoS genetics*, 15(11), e1008420.

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, 31(19), 3210-3212.

Simonson, T. S., Yang, Y., Huff, C. D., Yun, H., Qin, G., Witherspoon, D. J., Bai, Z., Lorenzo, F., P., Xing, J., Jorde, L. B., Prchal, J. T. & Ge, R. (2010). Genetic evidence for high-altitude adaptation in Tibet. *Science*, 329(5987), 72-75.

Sinervo, B., Mendez-De-La-Cruz, F., Miles, D. B., Heulin, B., Bastiaans, E., Villagrán-Santa Cruz, M., Lara-Resendiz R., Martinez-Mendez N., Calderon-Espinosa M. L., Meza-Lazaro, R. N., Gadsden, H., Avila, L., Morando, M., De La Riva, I., Sepulveda, P., Rocha, C., Ibarquengoytia, N., Puntriano, C., Massot, M., Lepetz, V, et al. & Sites, J. (2010). Erosion of lizard diversity by climate change and altered thermal niches. *Science*, 328(5980), 894-899

Smit, A. F. A., Hubley, R., & Green, P. (2013). RepeatMasker Open-4.0. Available from: <http://www.repeatmasker.org>. Accessed at 7th December 2021 through GenSAS.

- Solano, L. A. R., Navas, C. A., Carvajalino-Fernández, J. M., & Amézquita, A. (2016). Thermal ecology of montane *Atelopus* (Anura: Bufonidae): A study of intrageneric diversity. *Journal of thermal biology*, 58, 91-98.
- Song, S., Yao, N., Yang, M., Liu, X., Dong, K., Zhao, Q., Pu, Y., He, X., Guan, W., Yang, N., Ma, Y. & Jiang, L. (2016). Exome sequencing reveals genetic differentiation due to high-altitude adaptation in the Tibetan cashmere goat (*Capra hircus*). *BMC genomics*, 17(1), 1-12.
- Souchet, J., Gangloff, E. J., Micheli, G., Bossu, C., Trochet, A., Bertrand, R., Clobert, J., Calvez, O., Martinez-Silvestre, A., Darnet, E., Le Chevalier, H., Guillaume, O., Mossol-Torres, M., Barthe, L., Pottier, G., Philippe, H., & Aubret, F. (2020). High-elevation hypoxia impacts perinatal physiology and performance in a potential montane colonizer. *Integrative Zoology*, 15(6), 544-557.
- Spencer, H. L., Jover, E., Cathery, W., Avolio, E., Rodriguez-Arabaolaza, I., Thomas, A. C., Alvino, V. V., Sala-Newby, G., Dang, Z., Fagnano, M., Reni, C., Rowlinson, J., Vono, R., Spinetti, G., Beltrami, A. P., Gargioli, C., Caporali, A., Angelini, G., & Madeddu, P. (2019). Role of TPBG (trophoblast glycoprotein) antigen in human pericyte migratory and angiogenic activity. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 39(6), 1113-1124.
- Stanke, M., Keller, O., Gunduz, I., Hayes, A., Waack, S., & Morgenstern, B. (2006). AUGUSTUS: ab initio prediction of alternative transcripts. *Nucleic acids research*, 34(suppl_2), W435-W439.
- Storz, J. F. (2021). High-altitude adaptation: mechanistic insights from integrated genomics and physiology. *Molecular biology and evolution*, 38(7), 2677-2691.
- Storz, J. F., & Scott, G. R. (2019). Life ascending: mechanism and process in physiological adaptation to high-altitude hypoxia. *Annual review of ecology, evolution, and systematics*, 50, 503-526.
- Sun, Y. B., Fu, T. T., Jin, J. Q., Murphy, R. W., Hillis, D. M., Zhang, Y. P., & Che, J. (2018). Species groups distributed across elevational gradients reveal convergent and continuous genetic adaptation to high elevations. *Proceedings of the National Academy of Sciences*, 115(45), E10634-E10641.
- Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3), 585-595.

- Thornton, P. K., Ericksen, P. J., Herrero, M., & Challinor, A. J. (2014). Climate variability and vulnerability to climate change: a review. *Global change biology*, 20(11), 3313-3328.
- Thorpe, R. S., & Richard, M. (2001). Evidence that ultraviolet markings are associated with patterns of molecular gene flow. *Proceedings of the National Academy of Sciences*, 98(7), 3929-3934.
- Thorvaldsdóttir, H., Robinson, J. T., & Mesirov, J. P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefings in bioinformatics*, 14(2), 178-192.
- Velotta, J. P., Robertson, C. E., Schweizer, R. M., McClelland, G. B., & Cheviron, Z. A. (2020). Adaptive shifts in gene regulation underlie a developmental delay in thermogenesis in high-altitude deer mice. *Molecular biology and evolution*, 37(8), 2309-2321.
- Wang, H. B., Zan, L. S., & Zhang, Y. Y. (2014). Profiling of the yak skeletal muscle tissue gene expression and comparison with the domestic cattle by genome array. *Animal*, 8(1), 28-35.
- Wang, L., Bi, R., Yin, H., Liu, H., & Li, L. (2019). ENO1 silencing impairs hypoxia-induced gemcitabine chemoresistance associated with redox modulation in pancreatic cancer cells. *American Journal of Translational Research*, 11(7), 4470.
- Wang, X., Liu, S., Zuo, H., Zheng, W., Zhang, S., Huang, Y., Pingcuo G., Ying, H., Zhao, F., Li, Y., Liu, J., Yi, T. S., Zan, Y., Larkin, R. M., Deng, X., Zeng, X., & Xu, Q. (2021). Genomic basis of high-altitude adaptation in Tibetan *Prunus* fruit trees. *Current Biology*, 31(17), 3848-3860.
- Weisenfeld, N. I., Kumar, V., Shah, P., Church, D. M., & Jaffe, D. B. (2017). Direct determination of diploid genome sequences. *Genome research*, 27(5), 757-767.
- Witt, K. E., & Huerta-Sánchez, E. (2019). Convergent evolution in human and domesticated adaptation to high-altitude environments. *Philosophical Transactions of the Royal Society B*, 374(1777), 20180235.
- Wu, J., Yu, Z., & Su, D. (2014). BMP4 protects rat pulmonary arterial smooth muscle cells from apoptosis by PI3K/AKT/Smad1/5/8 signaling. *International journal of molecular sciences*, 15(8), 13738-13754.
- Yang, J., Jin, Z. B., Chen, J., Huang, X. F., Li, X. M., Liang, Y. B., Mao, J. Y., Chen, X., Zheng, Z., Bakshi, A., Zheng, D. D., Zheng, M. Q., Wray, N. R., Visscher, P. M., Lu, F.,

- & Qu, J. (2017a). Genetic signatures of high-altitude adaptation in Tibetans. *Proceedings of the National Academy of Sciences*, 114(16), 4189-4194.
- Yang, W., Qi, Y., & Fu, J. (2014). Exploring the genetic basis of adaptation to high elevations in reptiles: a comparative transcriptome analysis of two toad-headed agamas (genus *Phrynocephalus*). *PLoS One*, 9(11), e112218.
- Yang, W., Qi, Y., & Fu, J. (2016). Genetic signals of high-altitude adaptation in amphibians: a comparative transcriptome analysis. *BMC genetics*, 17(1), 1-10.
- Yang, W., Qi, Y., Lu, B., Qiao, L., Wu, Y., & Fu, J. (2017b). Gene expression variations in high-altitude adaptation: a case study of the Asiatic toad (*Bufo gargarizans*). *BMC genetics*, 18(1), 1-8.
- Yurchenko, A. A., Recknagel, H., & Elmer, K. R. (2020). Chromosome-level assembly of the common lizard (*Zootoca vivipara*) genome. *Genome biology and evolution*, 12(11), 1953-1960.
- Žagar, A., Carretero, M. A., Marguč, D., Simčič, T., & Vrezec, A. (2018). A metabolic syndrome in terrestrial ectotherms with different elevational and distribution patterns. *Ecography*, 41(10), 1728-1739.
- Žagar, A., Simčič, T., Dajčman, U., & Megía-Palma, R. (2022): Parasitemia and elevation as predictors of hemoglobin concentration and antioxidant capacity in two sympatric lizards. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 270, 111233.
- Zhang, Q., Gou, W., Wang, X., Zhang, Y., Ma, J., Zhang, H., Zhang, Y & Zhang, H. (2016). Genome resequencing identifies unique adaptations of Tibetan chickens to hypoxia and high-dose ultraviolet radiation in high-altitude environments. *Genome biology and evolution*, 8(3), 765-776.

3.9. Supplementary materials

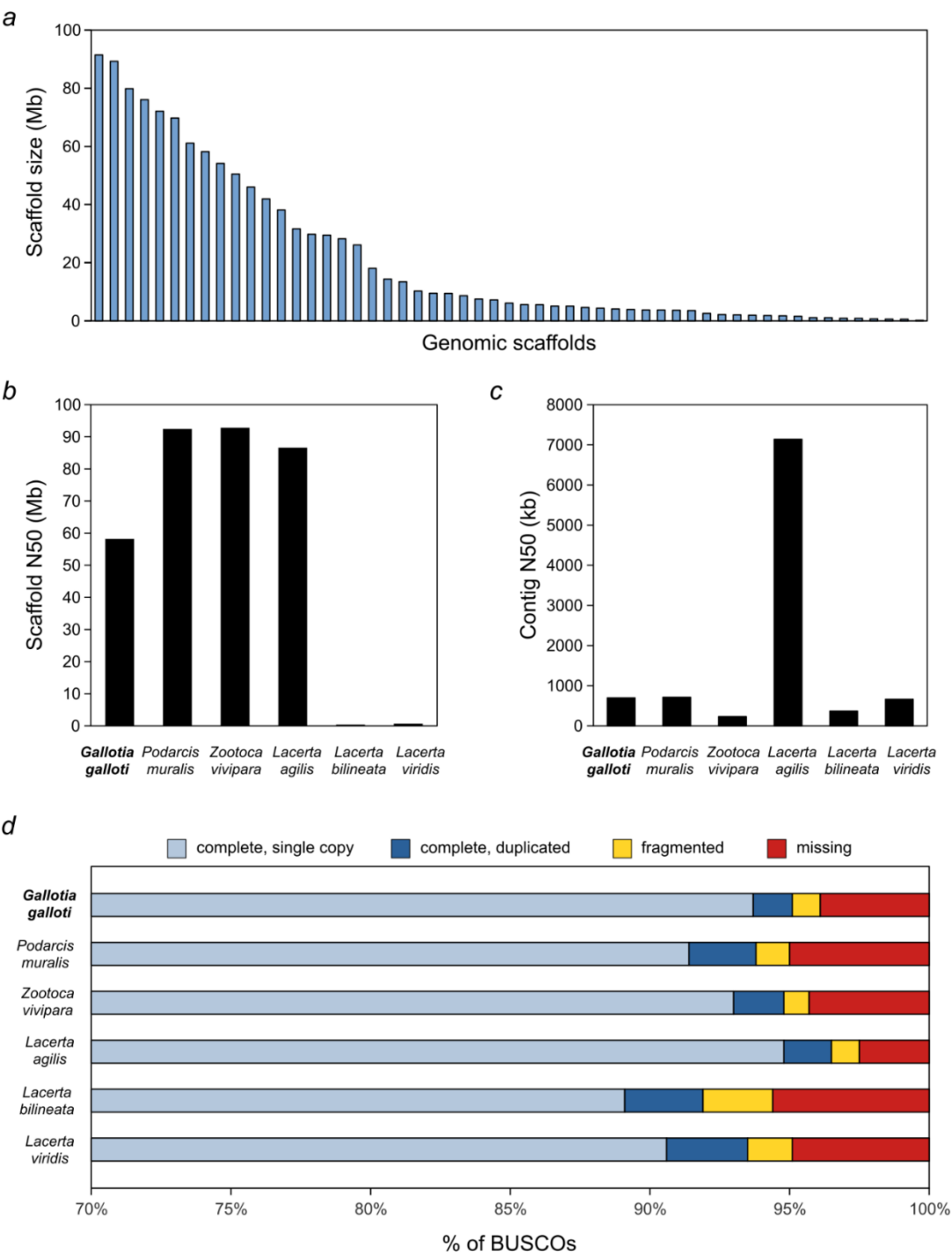


Figure S3.1. Comparison between the *Gallotia galloti* *de novo* assembly and other published lacertid genomes (*Lacerta agilis*, *Lacerta bilineata*, *Lacerta viridis*, *Podarcis muralis* and *Zootoca vivipara*). (a) Size (Mb) of the top 55 genomic scaffolds of *G. galloti* (all scaffolds larger than 100 kb). (b) Scaffold N50. (c) Contig N50. (d) BUSCO scores (aligned to the tetrapoda_odb10 database).

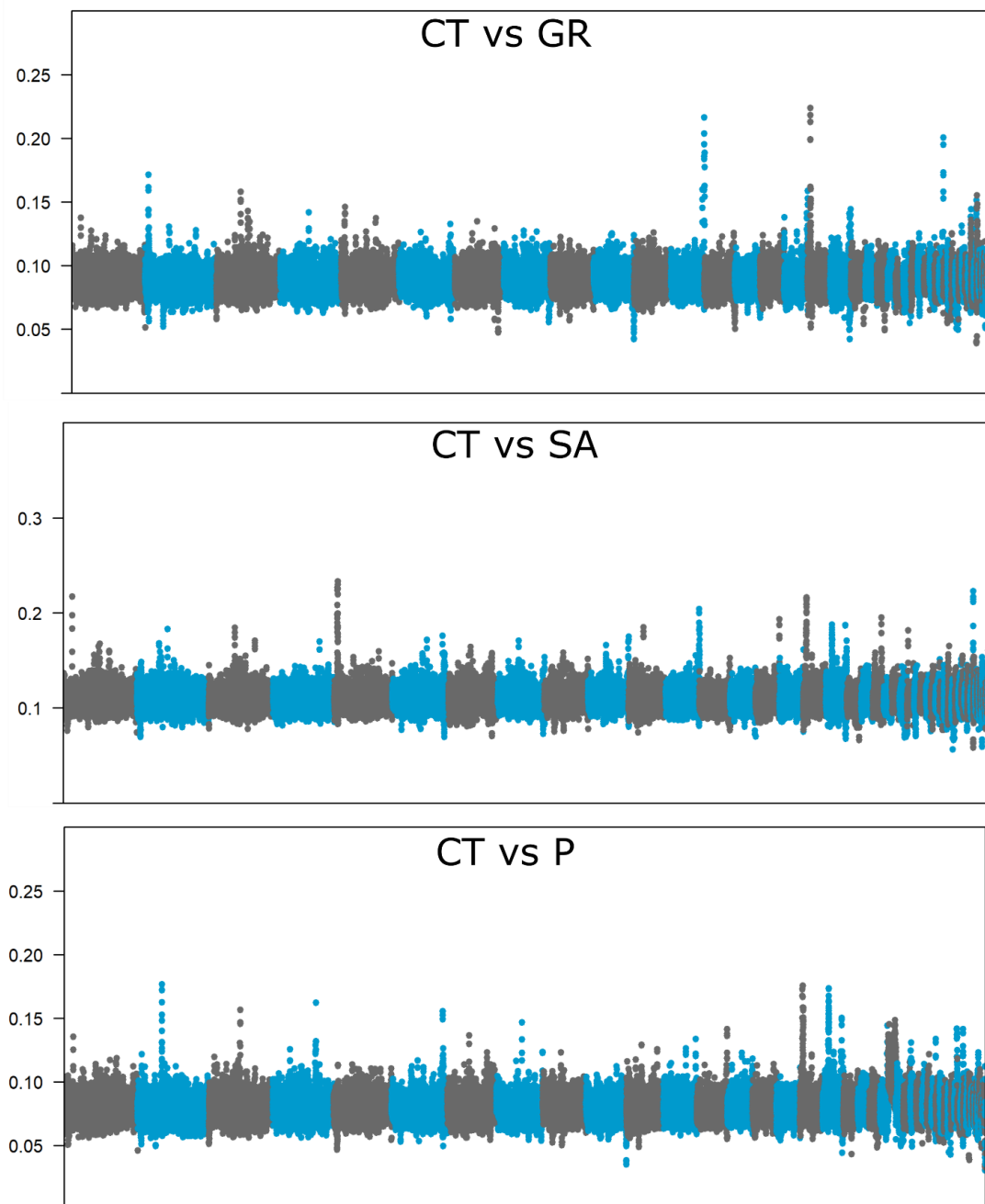


Figure S3.2. Genetic differentiation (fixation index, F_{ST}) between high-altitude (CT) and low-altitude (GR, P, SA) populations of *G. galloti*. Each dot corresponds to a 50 kb window, with successive windows having a 12.5 kb overlap. Alternating colors indicate the different genomic scaffolds.

Table S3.1. Summary statistics of the *de novo* reference genome assembly and annotation of *Gallotia galloti*.

CIBIO_Ggalloti_1.0	
Genome size (Gb)	1.18
Scaffold N50 (Mb)	58.15
Contig N50 (kb)	698.54
Number of scaffolds	7,039
Number of scaffolds >1 Mb	49
Number of scaffolds >100 kb	57
% GC	43.14
Complete single-copy BUSCOs (%)	7,006 (93.7%)
Complete duplicated BUSCOs (%)	103 (1.4%)
Fragmented BUSCOs (%)	74 (1.0%)
Missing BUSCOs (%)	297 (3.9%)

Table S3.2. Summary statistics of the whole-genome pool-sequencing dataset.

Population	Number of individuals in pool	Total number of reads	Percentage of duplicate reads	Coverage after duplicate removal	Percentage of mapped reads
CT (Cone of Teide)	14	285,764,025	67.0%	21.1	98.60%
GR (Granadilla de Abona)	16	113,667,347	50.5%	12.5	99.20%
SA (La Sabinita)	12	91,542,222	54.7%	9.2	99.00%
P (Porís de Abona)	12	141,685,537	51.9%	15.5	99.14%

Chapter IV – Ecophysiology and metabolic potential of two lizard lineages inhabiting contrasting habitats within an island

Ecophysiology and metabolic potential of two lizard lineages inhabiting contrasting habitats within an island

Nina Serén^{*1,2,3}, Rodrigo Megía-Palma^{1,4}, Tatjana Simčič⁵, Catarina Pinho^{1,2}, Anamarija Žagar^{1,5}, Miguel A. Carretero^{1,2,3}

¹*CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal*

²*BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal*

³*Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, 4099-002 Porto, Portugal*

⁴*Consejo Superior de Investigaciones Científicas (CSIC) Spain*

⁵*National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia*

4.1. Abstract

Environmental variation is an important driver of organismal diversification. When evolution is associated with adaptation to differing climates, insights into this process can be gained by examining how physiological and metabolic patterns vary in closely related lineages under different environmental conditions. In this study we focused on two lineages of the lizard *Gallotia galloti* which are distributed on opposing slopes of the island of Tenerife with contrasting microclimates. The north slope where *G. g. eisenrauti* occurs is more humid while in the south *G. g. galloti* occupies overall more arid environmental conditions. Our work, encompassing populations at different altitudes from both lineages, mainly showed variation between lineages in thermoregulatory preferences and patterns of evaporative water loss, supporting the expected pattern of microclimatic distribution. Compared to *G. g. galloti*, *G. g. eisenrauti* selects for lower temperatures, has different hourly patterns of thermal preferences and loses more water by evapotranspiration. Metabolic parameters are similar between subspecies, except for higher potential metabolic activity of *G. g. eisenrauti* at higher temperatures. Also, within each lineage, metabolism differs between populations sampled at different altitudes, but not in a linear way. The population of *G. g. eisenrauti* sampled close to the highest altitudinal range of this subspecies shows signs of greater physiological and metabolic stress. The physiological and metabolic differences between these two lineages are a potential explanation as to why only one of them, *G. g. galloti* has managed to colonize the extreme altitudinal environments of Tenerife.

Key-words: ecophysiology, preferred temperatures, evaporative water loss, microclimate, metabolic activity, altitudinal gradient

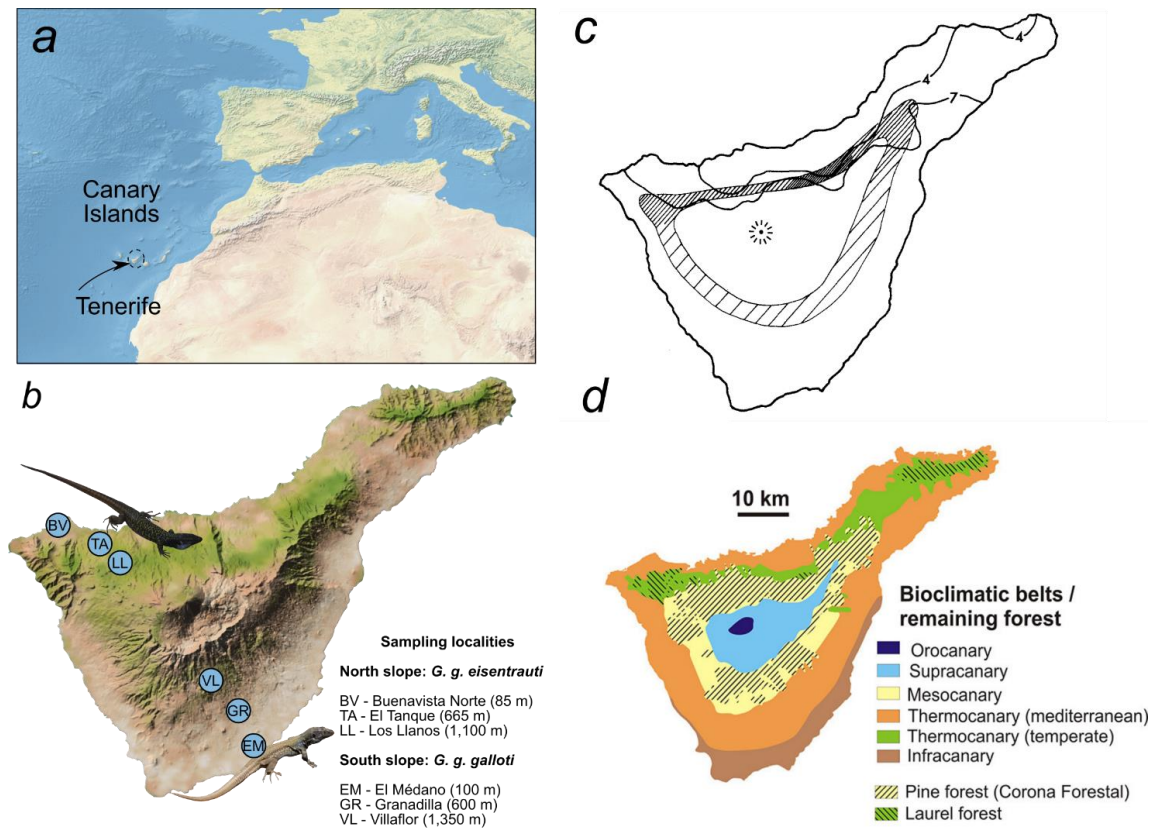
4.2. Introduction

Contrasting environments and resulting divergent natural selection are known drivers of biological diversification, and ultimately speciation (Rundle & Nosil, 2005; Schluter, 2009; Qvarnström et al., 2016). Although this observation is more readily obvious in lineages that become isolated by large geographic barriers, environmental variation at relatively smaller scales can also help drive divergent phenotypic evolution. It has also recently become widely accepted that even in the presence of gene flow, selection acting under these conditions can lead to contemporary evolution of reproductive isolation (Hendry et al., 2007; Smadja & Butlin, 2011). Under conditions of diversification associated with climate (either caused directly by it or indirectly associated), studies on the physiological requirements and metabolic potential of closely related species living in contrasting environments can provide us with clues on how climate shapes species' adaptation and diversification. This is particularly relevant for organisms that are potentially more vulnerable to climatic changes, such as lizards, given their relatively low mobility, which in theory should lead to a bigger reliance on coping strategies other than dispersal to deal with climate variability.

In this study we analyse how thermal physiology, hydric physiology and metabolism vary within and between two closely related lineages ranging continuously across contrasting environments within the same island. In the island of Tenerife (part of the archipelago of the Canary Islands, North Atlantic Ocean, Figure 4.1a) *G. galloti* is the most common and widespread species and is constituted of two major clades (Figure 4.1b): *G. galloti galloti* (herein simplified as *galloti*) occurs mostly around the Southern slope of Tenerife, while *G. galloti eisentrauti* (herein simplified as *eisentrauti*) is found in the island's North slope. Despite the restricted setting of the island of Tenerife, *galloti* and *eisentrauti* differ in their distribution, morphology, coloration and patterns of genetic variation (Bischoff, 1982; Molina et al., 1997; Thorpe & Richard, 1989, 2001; Bohórquez-Alonso, 2014, 2018; Brown et al., 2016). Both lineages inhabit a wide altitudinal gradient on either side of the island's main volcano, but the altitudinal distribution of *eisentrauti* is much more restricted than that of *galloti*, which can be found on top of the Teide volcanic cone (maximum elevations of around 1,200 and 3,700 metres, respectively). The evolutionary history of the two lineages, and how this relates to different stages in the geologic and paleoclimatic history of Tenerife still requires further analyses, particularly since divergence dates have not been estimated; however, within-*galloti* mitochondrial

divergence has been dated at 0.8 Mya (Brown et al., 2006), providing a likely lower boundary for the *galloti-eisentrauti* split.

The genesis of Tenerife lies in the formation of three paleoislands at around 11.6 Mya (Carracedo et al., 2002), which were joined into the current main island through multiple volcanic events that started around 2 Mya (Ancochea et al., 1990), with its subsequent geological history marked by several volcanic events, in particular recent episodes of volcanic activity that contributed to the construction of the Teide cone (Ancochea et al., 1990; Carracedo et al., 2002, 2007). The island's current climate is highly complex and diversified, consisting of several bioclimatic belts that are partly explained by the volcanic topography and predominant north-west winds that accumulate moist air currents and form “cloud-banks” on the North-facing slopes, leading to a cooler and more humid climate on this side, contrasting to a hotter and drier Southern slope of the island (Rivas-Martínez, 1987; Del Arco et al., 2006, Kunkel, 2012, see Figure 4.1c, 4.1d). We thus hypothesize that thermal and hydric ecophysiology (preferred temperatures and evaporative water loss rates, which are good proxies of physiological adaptation to climate, Garcia-Porta et al., 2019) and metabolism (metabolic potential and catalase antioxidant activity, with known direct relevance to fitness, Glazier et al., 2015) of *galloti* and *eisentrauti* will reflect the very different climatic regimes of their distribution. We additionally hypothesize that elevation may create additional complexity, given the sudden climatic gradients associated with the steep volcanic slope of the main volcanic edifice; if functional parameters follow this environmental variation irrespective of lineage, this could suggest adaptation or plasticity (if not, this could suggest conservativeness in function).



4.3. Methods

Sampling

Samples of *galloti* and *eisentrauti* were collected in two field missions to Tenerife held on July-August 2017 and 2018, coinciding with the end of the reproductive season of *galloti* and *eisentrauti* (Salvador, 2015). Both sampling years confirmed to the typical climate patterns for this season in Tenerife and did not differ substantially (<https://weatherspark.com>). The sampling design implemented two short altitudinal transects encompassing three locations for each lineage (Figure 4.1b). Sampling was restricted to adult males to avoid ecophysiology effects associated with age or sex (e.g. Carretero, 2006) and sampled between 9 and 19 lizards per location. Lizards were collected by noose (García-Muñoz & Sillero, 2010) or using tomato and banana as bait in pitfall traps. Elevation and the coordinates for each sampling site was registered with a GPS device (GPSMAP 64s, Garmin®, Kansas, USA). For each lizard, snout-vent length (SVL, ± 0.01 mm) and weight (± 0.0001 g) were measured. Adult males were carried to local laboratory facilities (Instituto de Productos Naturales y Agrobiología, IPNA, CSIC, elevation of 560 m a.s.l) to acclimatize in individual gradient terraria with water and food *ad libitum* for 12h. After ecophysiological testing, the tail tip tissue for metabolic analyses was retrieved (snap frozen in liquid nitrogen). All lizards were released in the same location of trapping. All ecophysiological and metabolic assays were performed using the same protocols as described in Chapter II of this thesis.

Thermal preferences experiments

Experiments to determine preferred body temperature (T_p) were performed with linear thermal gradients following the method in Koziel et al. (2021) and Veríssimo & Carretero, 2019. Briefly, unsheltered terraria were mounted with PVC opaque walls and sandy soil as substrate to allow the lizard to freely select the most adequate temperature throughout the experimentation time without thermoregulatory constraints (Veríssimo & Carretero, 2009, Supplementary Figure S4.3). A 150 W infrared lamp suspended above one end of the terraria was used as heat source to generate a thermal gradient ranging between 19 °C to 68 °C (preheated circa 1h before testing). Trials of lizards from different localities were also randomized. Lizards were kept under normal light photoperiod (with natural light) in a room at around 24 °C (Koziel et al., 2021). Each lizard was randomly assigned to an individual terrarium at around 19:00 on the evening before the test day.

Cloacal temperature measurements were performed every hour between 8:00-17:00 (WEST time zone) using a type-k thermocouple HIBOK® 18 (precision 0.1 °C).

Evaporative water loss experiments

Experiments of evaporative water loss (EWL) were performed the day after the preferred body temperature assays. In the intervening night, lizards were provided with a humid cloth to hydrate. 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 (48x38x30 cm) under constant humidity conditions (~25%) maintained by approximately 150 g of silica gel placed in a fabric net glued under the chamber lid (following the protocol of Ferreira et al., 2016). Instantaneous water loss (EWL_i) values were obtained from an initial weight measurement recorded before the start of the experiment (W₀) and hourly records of weight (W_n) using this formula: $((W_n - W_{n+1})/W_n) \times 100$. The average water loss at different end points of the experiment (EWL_{s-e}) was also calculated for an increasing duration of the experiment, obtaining 11 EWL_{s-e} values (calculated from 2, 3, 4 ... 12 EWL_i values) and EWL_a using the formula: $EWL_a = [W_0 - W_n]/W_0$. For subsequent analyses, both the instantaneous water loss (EWL_i) relative to initial weight, W₀; using the formula $EWL_i = [(W_n - W_{n+1})/W_0] \times 100$; and the accumulated water loss (EWL_a) were examined in each time interval for each repeated trial measure. EWL_i captures the amount of EWL in each moment whereas EWL_a provides a measurement of the water lost by lizards throughout the experiment.

Potential metabolic activity

To test for potential metabolic activity (PMA) approximately 10 mm length from snap frozen tail tip tissue was cut and homogenized with the help of a mortar and liquid nitrogen. The moist homogenized tissue was then weighted, and approximately 25 mg of wet weight was sonicated in 3 mL of ice-cold homogenization buffer 0.1 M sodium phosphate buffer pH = 8.4; 75 µM MgSO₄; 0.15% (w/v) polyvinyl pyrrolidone; 0.2% (v/v) Triton-X-100) for 20 s (Cole-Parmer® ultrasonic homogenizer 4710) followed by a 4 min centrifugation at 0 °C and 10,000 rotations per minute (Centrifuge SIGMA® 2K15). Two replicates of 30 µL homogenate of the same sample and one blank without the homogenate were incubated simultaneously in plates filled with 150 µL of 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 of 2.5 mM 2-(p-iodophenyl)-3-(pnitrophenyl)-5-phenyl tetrazolium chloride (INT) solution). The incubation was carried out in parallel at three different incubator chambers during 20 minutes with three different temperature regimes (29 °C, 33 °C and 37 °C) around the mean preferred body temperature, which in *G. galloti* was $T_p = 33.84$ °C (see results). The reaction was terminated with 50 μL of stopping solution (formalin: H_3PO_4 concentration = 1:1 v/v). The final step to verify formazan production of the enzymatic process was determined spectrophotometrically from the absorbance of the sample at 490 nm against the control (microplate reader BioTek Instruments SynergyMX). From the spectrophotometry values, standard calculations were performed following Kenner and Ahmed (1975) to obtain a coarse estimate of PMA. The values of PMA were estimated as the INT reduction capacity ($\mu\text{L O}_2 \text{ mg}^{-1} \text{ protein h}^{-1}$) following the formula: $\text{INT reduction capacity} = (\text{ABS}_{490} \times \text{Vr} \times \text{Vh} \times 60 \times 1.30) / (\text{Va} \times \text{S} \times \text{t} \times 1.42)$; where ABS_{490} is the absorption spectrum of the sample at a wavelength of 490 nm; 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 O_2 (Kenner and Ahmed, 1975).

Catalase activity

Catalase activity was assessed following the method of Aebi (1984). 0.3 mL of the previously obtained tissue homogenate was mixed with 1.7 mL of 50 mM potassium phosphate buffer pH 7.0. Additionally, 1 mL of 30 mM hydrogen peroxide, prepared in 50 mM potassium phosphate buffer pH 7.0, was added to obtain a final concentration of hydrogen peroxide of 10 mM. Spectral analysis of the decomposition of the hydrogen peroxide was done by measuring absorbance at every 30 s at a wavelength of 240 nm for 3 min at 25 °C using quartz cuvettes with a path length of 10 mm and a Lambda UV/Vis spectrophotometer (PerkinElmer, Waltham, MA, USA). Enzyme activities were expressed in enzyme units (U), in which one U is the amount of catalase that degrades one μmol of hydrogen peroxide in 1 min ($\epsilon_{240} = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$). These results were then divided by the total amount of protein to give the specific catalase activity per mg protein. Total protein concentration of the homogenate was assessed with a Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) using a microplate reader following the manufacturer's instructions.

Statistical analyses

All analyses were conducted in *R* v4.2 (R Core Team, 2022), unless otherwise noted. To account for effects of size, weight and robustness, analyses of two-way variance (2-way ANOVA) and of covariance (ANCOVA) were performed using *aov* function in *R*. Populations of each lineage that were sampled at different altitudes were not nested within a higher “lineage” factor, to allow for altitude to be considered as a separate factor (altitude was thus tested in the pool of both lineages together). Standardized measures were transformed using a log10 transformation. Allometric effects of body size (SVL used as proxy) were removed from physiological and biochemical variables using Thorpe-Lleonart’s method (Lleonart et al., 2000, as implemented in *PAST* v4.10 Hammer et al., 2001). Extreme outliers for each dataset were identified using Tukey’s method (*identify_outliers* function in *rstatix* v.0.7.0 package in *R*) and removed from subsequent analyses. Final sample sizes for analysed variables per location are discriminated in Table 4.1. Normality was assessed using Shapiro-Wilk’s tests; most variables were not significantly different 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 from homoscedasticity). Assumption of sphericity for variables with repeated measures was checked with Mauchley’s tests (no significant deviations in any variable).

The main objective of all analyses was to check for significant differences in physiological and biochemical variables between lineages and different sampled altitudes. Differences between populations in variables without repeated measurements (catalase activity) were assessed using 2-way ANOVA, *aov* function in *R*, and post-hoc pairwise differences using t-tests were performed using the function *pairwise.t.test* in *R*, with the *P* value adjusted with Benjamini-Hockberg procedure to reduce the false discovery rate at $P < 0.05$. 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) a three-way repeated measures ANOVA (3-way RM-ANOVA, *anova_test* function in *rstatix* v.0.7.0 package in *R*) was implemented with lineage and altitude as between-subject factors and time-point/temperature as a within-subject factor. Significance levels across the different analyses were considered at a threshold of $P < 0.05$.

Table 4.1 – Descriptive statistics of the collected data of populations from different sampling localities. Sample size shown for the analyses of thermogradient preferences (T_p) and accumulated evaporative water loss (EWL_a) include the number of individuals after outlier removal.

Lineage	Location / altitude (m)	Sample sizes (SVL / T_p , / W_0 / EWL_a / PMA / Catalase)	SVL (mm) Mean $\pm$ SD / Min-Max	T_p ($^{\circ}$ C) Mean $\pm$ SD /Min-Max	W_0 (g) Mean $\pm$ SD /Min-Max	EWL (mg) Mean $\pm$ SD /Min-Max	PMA (33 $^{\circ}$ C) Mean $\pm$ SD /Min-Max	Catalase (U/mg protein) Mean $\pm$ SD / Min-Max
<i>G. g. galloti</i>	EM / 100m	15 / 14 / 18 / 18 / 14 / 15	99.7 $\pm$ 7.68 / 85-109	34.2 $\pm$ 1.82 / 31.8-37.8	29.2 $\pm$ 6.74 / 20.21-42.23	0.66 $\pm$ 0.357 / 0.25-1.59	4.79 $\pm$ 1.333 / 3.08-7.53	6.03 $\pm$ 2.046 / 2.66-10.82
<i>G. g. galloti</i>	GR / 600m	17 / 16 / 18 / 18 / 16 / 17	108.6 $\pm$ 9.58 / 92-123	34.1 $\pm$ 1.50 / 32.6-35.7	40.0 $\pm$ 8.91 / 25.62-57.64	0.83 $\pm$ 0.447 / 0.25-1.16	6.32 $\pm$ 1.933 / 2.18-9.13	6.97 $\pm$ 2.462 / 1.85-10.65
<i>G. g. galloti</i>	VL / 1350m	16 / 13 / 16 / 16 / 17 / 17	103.2 $\pm$ 4.51 / 93-108	33.7 $\pm$ 1.58 / 32.4-35.4	35.14 $\pm$ 5.32 / 25.9-45.3	0.70 $\pm$ 0.467 / 0.25-1.18	5.33 $\pm$ 1.580 / 2.97-8.01	7.83 $\pm$ 3.857 / 3.01-14.84
<i>G. g. eisentrauti</i>	BV / 100m	18 / 17 / 19 / 19 / 18 / 18	121.1 $\pm$ 6.05 / 111-135	31.6 $\pm$ 1.93 / 27.8-34.0	53.8 $\pm$ 8.43 / 40.7-69.3	0.73 $\pm$ 0.292 / 0.37-1.14	4.98 $\pm$ 1.304 / 3.21-8.78	7.25 $\pm$ 3.118 / 1.42-14.24
<i>G. g. eisentrauti</i>	TA / 650m	12 / 13 / 13 / 13 / 11 / 12	118.1 $\pm$ 11.64 98 / 137	32.0 $\pm$ 1.81 / 30.6-33.2	52.4 $\pm$ 19.12 / 27.5-93.3	1.14 $\pm$ 0.398 / 0.63-2.17	5.42 $\pm$ 1.380 / 2.82-7.17	7.12 $\pm$ 1.890 / 4.63-10.15
<i>G. g. eisentrauti</i>	LL / 1100m	8 / 9 / 9 / 9 / 8 / 9	107.7 $\pm$ 6.84 / 96-120	31.5 $\pm$ 1.99 / 27.4-33.6	38.2 $\pm$ 8.18 / 20.7-46.4	1.05 $\pm$ 0.406 / 0.60-1.93	5.35 $\pm$ 1.082 / 3.96-7.12	7.30 $\pm$ 1.478 / 4.72-9.74

4.4. Results

Body size comparison

The two lineages differed significantly in SVL, weight and robustness (weight accounting for SVL), with higher values of these measures in *eisentrauti* (Table 4.2, Supplementary Figure S4.1, S4.2; Supplementary Tables S4.1, S4.2). SVL and weight diverged between sampling locations at different altitudes, particularly in *eisentrauti*, since its population at the highest altitude (LL) is relatively smaller and lighter compared to all other populations. In *galloti* the only statistically different pairwise difference was found between EM and GR.

Thermal preferences

A direct visualization of the hourly patterns of variation in T_p (non-transformed and uncorrected for SVL) between subspecies suggested differences in their thermal ecology, but dependent on time rather than on average values (Supplementary Figure S4.1). When we corrected these variables for size and test for differences using 3-way RM-ANOVA we confirmed significant effects in thermal preferences between lineages, as well as differences between populations at different altitudes – irrespectively of lineage (in particular the mid and higher elevation) and between time of the day (Table 4.2). Overall, thermal preferences tended to follow a U-shape (lizards regulated for lower temperatures in the middle of the day, when external temperatures should be higher). However, one must account for the significant interaction between lineages and time of the day, meaning that each lineage had a different thermal preference pattern throughout the day. This effect was mostly driven by the data from two populations of *eisentrauti* which do not followed the general U-shape pattern, indicating instead a drop in T_p throughout the day, even before sunset (Figure 4.2a).

Evaporative water loss

An overview of the temporal variation of raw EWL data with non-transformed variables suggested differences between lineages in their hydric physiology, in particular when looking at patterns of EWL_a , for which differences gradually increased with time (Supplementary Table 4.3). 3-way RM-ANOVA of the size-corrected variables for both EWL_i and EWL_a revealed significant differences in water-loss patterns between time of

the day, lineages and altitude – irrespectively of lineage (here only for EWL_a) (Table 4.2, Figure 4.2c). Detailed inspection indicated that water loss was higher for *eisentrauti*, and within this lineage it was higher at the mid-elevation population (Figure 4.2c). A significant interaction was found for EWL_i for factors altitude and time of the day. This interaction is explained because for both populations of both lineages at different altitudes there is a difference in their hourly pattern of EWL_i.

Metabolism and antioxidant capacity

PMA was significantly different between altitudes: differences between low-altitude against both mid-altitude *galloti* and mid-altitude *eisentrauti*. Comparison of the PMA across three temperature regimes revealed that in *galloti* there is a linear increase with temperature, while in *eisentrauti* PMA increased more substantially between middle and highest temperature than between lowest and middle temperature, which is evidenced by a significant interaction between the subspecies and tested temperatures (Table 4.2, Figure 4.2d). The 2-way ANOVA for catalase activity did not reveal significant differences between either lineages or altitude (Table 4.2).

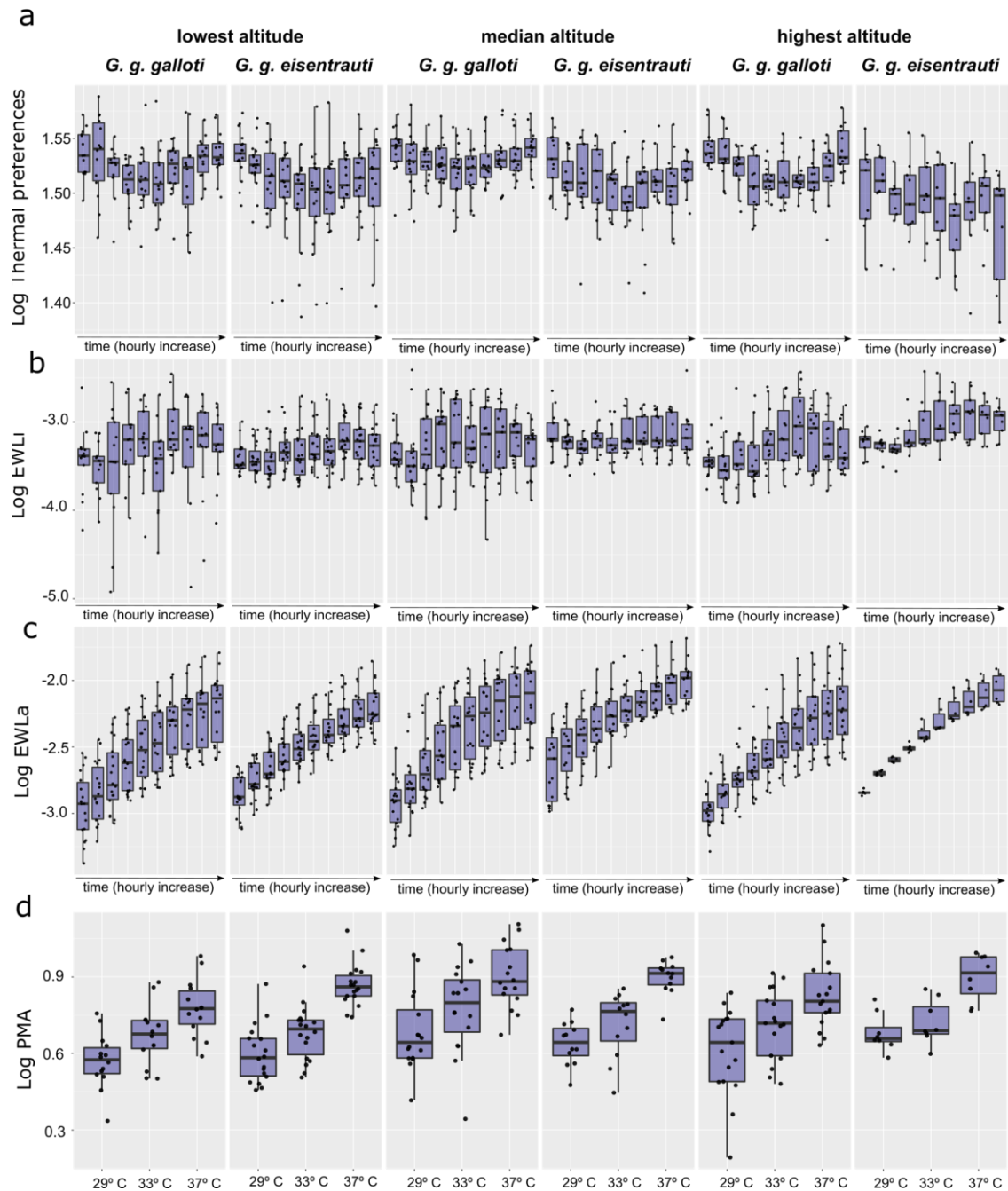


Figure 4.2. Box-and-jitter plots showing patterns of: (a) thermal preferences (T_p) along different periods of the day by lineage and altitude; (b) accumulated water loss (EWL_a) along the different periods of the day by lineage and elevation; (c) instantaneous water loss (EWL_i) along the different periods of the day; (d) potential metabolic activity (PMA) along three distinct testing temperatures (29 °C, 33 °C and 37 °C) by lineage and altitude.

Table 4.2. Results of different analyses of variance (ANOVA) to assess variation in ecophysiological and metabolic traits in relation to subspecies and relative elevation between sampled locations. Abbreviations: T_p : preferred temperature; EWL_i : instantaneous evaporative water loss; EWL_a : accumulated evaporative water loss; PMA: potential metabolic activity. Showing Generalized eta squares for Repeated Measures ANOVA and Sum squares for ANCOVA and 2-way ANOVA.

	Effect	Total df	DFn	DFd	F	Generalized eta or Sum squares	P value
3-way Repeated Measures ANOVA	T_p	Lineage	1	76	28.509	0.122	< 0.001
		Altitude	2	76	3.887	0.036	0.02
		Time	7.44	565.8	10.085	0.077	< 0.001
		Lineage x Altitude	2	76	1.644	0.016	0.200
		Lineage x Time	7.44	565.8	2.845	0.023	0.005
		Altitude x Time	14.89	565.8	1.084	0.018	0.369
		Lineage x Altitude x Time	14.89	565.8	0.898	0.015	0.566
	EWL_i	Lineage	1	80	4.123	0.026	0.046
		Altitude	2	80	2.980	0.037	0.056
		Time	6.06	484.75	12.880	0.072	< 0.001
		Lineage x Altitude	2	80	1.260	0.016	0.289
		Lineage x Time s	6.06	484.75	1.348	0.008	0.234
		Altitude x Time	12.12	484.75	1.770	0.021	0.050
		Lineage x Altitude x Time	12.12	484.75	1.113	0.013	0.347
	EWL_a	Lineage	1	86	6.838	0.062	0.010
		Altitude	2	86	5.304	0.093	0.007
		Time	1.65	142.19	553.473	0.527	< 0.001
		Lineage x Altitude	2	86	1.264	0.024	0.288
		Lineage x Time	1.65	142.19	2.292	0.005	0.140
		Altitude x Time	3.31	142.19	1.107	0.004	0.351
		Lineage x Altitude x Time	3.31	142.19	0.504	0.002	0.698
	PMA	Lineage	1	79	0.563	0.006	0.455
		Altitude	2	79	3.463	0.066	0.036
		Temperature	1.78	140.83	250.053	0.380	< 0.001
		Lineage x Altitude	2	79	1.266	0.025	0.288
		Lineage x Temperature	1.78	140.83	5.165	0.013	0.009
		Altitude x Temperature	3.57	140.83	0.291	0.001	0.864
		Lineage x Altitude x Temperature	3.57	140.83	0.428	0.004	0.766
ANCOVA	Robustness	Weight	1		263.521	0.058	< 0.001
		Altitude	5		3.275	0.004	< 0.001
		Residuals	77			0.017	-
2-way ANOVA	SVL	Lineage	1		96.33	0.097	< 0.001
		Altitude	4		4.49	0.018	0.002
		Residuals	78			0.097	
	Weight	Lineage	1		39.237	0.323	< 0.001
		Altitude	4		5.136	0.169	0.001
		Residuals	78			0.642	
	Catalase	Lineage	1	4	25.271	-	0.474
		Altitude	2		0.832		0.439
		Lineage x Altitude	82		0.080		0.923

4.5. Discussion

Studying how closely-related lineages respond to climatic differences is crucial to better understand how organisms may cope under scenarios of future climate change. For ectothermic vertebrates, this is important because in general these are organisms that are vulnerable to shifts in climates. In this context, systems like *Gallotia galloti galloti* and *G. g. eisenrauti*, two closely related lizard lineages that inhabit the island of Tenerife, are particularly interesting since they comprise sister taxa that are presently restricted to areas with different microclimates within a very short spatial range (two sides of the same mountain). In our work we show that lineages differ in their thermal preferences, patterns of evaporative water loss and metabolic potential. Specifically, preferred temperatures diverged between lineages, not only in their overall values, but also in how they select for different temperatures throughout the day (*eisenrauti* selects for lower temperatures overall, and decreases its preferred temperature throughout the day). Rates of evaporative water loss were higher for *eisenrauti* than for *galloti*. Metabolic potential and catalase activity were similar between lineages, but metabolic potential had a greater rate of variation between lineages at higher test temperatures, suggesting that higher temperatures disproportionately affect *eisenrauti* more than *galloti*.

In accordance with the general observation of both *G. gallotia* lineages having different morphological characteristics (Bischoff, 1982; Thorpe & Brown, 1989; Thorpe & Richard, 2001), size and robustness were markedly different, with *eisenrauti* being larger than its southern sister lineage. Differences in some of the populations (the highest of the *eisenrauti* with lowest size and the lowest from *gallotia* with lowest weight) may be related to local microclimatic conditions or edge-distribution effects that can cause suboptimal conditions (or parasite load at the most low-altitude southern populations, see Megía-Palma et al., 2020).

The marked differences in thermal and hydric physiology of the two lineages are likely linked to the different microclimates experienced on each side of Tenerife, however detected differences across altitude (irrespective of lineage) also hint at changed environmental conditions. Thus, both effects (lineage and altitude) are likely contributing to observed variation in studied traits. These important ecophysiological traits are directly related to fitness (e.g. Huey & Bennet, 1987; Bauwens et al., 1995; Wang et al., 2016; Zani & Stein, 2018), and typically show high conservation (particularly preferred temperatures, since hydric ecology seems more flexible according to local environment), so they tend to show similar patterns in populations of the same species inhabiting

different climate regimes (Van Damme et al., 1989; Van Damme et al., 1990; Tosini & Avery, 1993; Gvozdík & Castilla, 2001; Carretero et al., 2005). The southern *galloti* inhabits typically hotter and more arid habitats, so the higher preferred temperatures and reduced evaporative water loss match the expectations of lacertids adapted to drier environments, with the opposite effects for *eisentrauti* (Cox et al., 2015). Apart from different average preferred temperatures, *eisentrauti* also tend to have an alternative hourly profile in their thermal preferences: while *galloti* exhibit a U-shaped pattern (selecting for higher temperatures at the beginning and end of the day), *eisentrauti* (particularly at mid and higher altitudes) tend to also downregulate their temperature at the end of the day (potentially linked to their hydric physiology, as suggested by S'khifa et al., 2020). Similarly to *eisentrauti*, higher rates of water loss have also been linked to microclimate in an altitudinal gradient in an insular lacertid (Belasen et al., 2017). The contrasting microclimates of Tenerife are thus likely to have been an additional contributing factor in shaping the distribution and divergence patterns of the *G. galloti* subspecies that occur in the island (Thorpe & Richard, 1989), since they create very specific conditions that allow alternative adaptive strategies to arise. This may limit gene flow between the lineages, although the relative importance of this and other potential pre-zygotic barriers such as mating behaviour or colourful ornaments is still unknown (Thorpe & Richard, 1989, 2001; Bohórquez-Alonso, 2014, 2018).

Although we did get some significant differences within lineages linked to populations inhabiting different altitudes, we generally did not get a linear signal associated with increasing altitudes. However, the population of *eisentrauti* inhabiting at higher altitudes (circa 1,200 m a.s.l.) showed a tendency for more divergent patterns in some variables: 1) it has a more pronounced deviation to the U-shaped pattern in hourly thermal regulation, and 2) it shows a stronger effect of the higher testing temperature on metabolism. This population lives at the altitudinal edge of the distribution of *eisentrauti*, coinciding with locations with constant cloud cover and an important shift in vegetation composition and structure, to dense forested Canarian pine (Rivas-Martínez, 1987), which together with the physiology and metabolism data suggest an intrinsic limitation in their capacity to colonize areas further upland. Both *galloti* and *eisentrauti* are widely distributed throughout their respective sides of Tenerife, but only *galloti* reaches locations with the highest altitudes, having been found up to the summit of the Teide volcano (~3,700 m a.s.l., Fariña et al., 2011, and visual confirmation during 2017 by NS, RM-P and AZ). Within *galloti* populations inhabiting these extreme elevations, thermal preferences and patterns of water loss tested in laboratory conditions are strikingly similar, pointing to conservatism in fundamental niche with a high degree of functional

plasticity in their physiological capacity to cope with different thermal environments (see Chapter II of this thesis). It is thus likely that the better performance of *gallotia* to drier habitats could have facilitated the colonization of the also arid highest altitudes habitats, something that could limit the expansion of the distribution of *eisentrauti* (arid areas in general seem to be linked to stress in this species, Megía-Palma et al., 2020). However, further testing is needed to get a more detailed understanding on their evolutionary history from a functional perspective. Altogether, these sources of data have the potential to expand on our earlier insights into how the ecophysiology and metabolism shape the adaptive capacity of lineages, which can ultimately inform us on their resilience to drastic environmental and climatic change.

4.6. Acknowledgements

NS was funded by a PhD scholarship (SFRH/BD/128612/2017) and extension FCT grant (COVID/BD/152580/2022) attributed by Fundação para a Ciência e Tecnologia. CP was supported by Portuguese national funds through FCT-Fundação para a Ciência e a Tecnologia in the scope of the project UIDB/50027/2020. MAC was supported by the project NORTE-01-0145-FEDER-000007. AŽ and TS were funded from the state budget by the Slovenian Research Agency (Programme P1-0255 and project grant J1-2466). This project is funded by a Portugal 2020 FCT project (2801402/SAICT/2017). We are thankful to Manuel Nogales, current director of IPNA-CSIC (Spanish National Research Council - Department of Agrobiology and the Environment) for all the support and providing an ecology lab to conduct the experiments at La Laguna (Tenerife, Spain). A very special thanks for precious help in the field work carried out in Tenerife, to Julien Piquet, Miha Krofel, Nayra Hernández-Acosta, Senda Reguera and Xavier Santos.

4.7. References

- Aebi, H. (1984) Catalase in Vitro. *Methods in Enzymology*, 105, 121-126.
- Ancochea, E., Fuster, J., Ibarrola, E., Cendrero, A., Coello, J., Hernan, F., Cantagrel, M. & Jamond, C. (1990). Volcanic evolution of the island of Tenerife (Canary Islands) in the light of new K-Ar data. *Journal of Volcanology and Geothermal Research*, 44(3-4), 231-249.
- Bauwens, D., Garland, T., Jr., Castilla, A.M., Van Damme, R. (1995). Evolution of sprint speed in lacertid lizards: morphological, physiological, and behavioural covariation. *Evolution*, 49, 848-863.
- Belasen, A., Brock, K., Li, B., Chremou, D., Valakos, E., Pafilis, P., Sinervo, B. & Foufopoulos, J. (2017). Fine with heat, problems with water: microclimate alters water loss in a thermally adapted insular lizard. *Oikos*, 126(3), 447-457.
- Bischoff, W. (1982): Die innerartliche Gliederung von *Gallotia galloti* (Dumeril & Bibron, 1839)(Reptilia: Sauria: Lacertidae) auf Teneriffa, Kanarische Inseln. *Bonner zoologische Beiträge*, 33(2-4), 363-382.
- Bohórquez-Alonso, M. L. and Molina-Borja, M. (2014). Reflectance of sexually dichromatic UV-blue patches varies during the breeding season and between two subspecies of *Gallotia galloti* (Squamata: Lacertidae). *Biological Journal of the Linnean Society*, 113, 556-569.
- Bohórquez-Alonso, M. L., Mesa-Ávila, G., Suárez-Rancel, M. M., Font, E. & Molina-Borja, M. 2018. Predictors of contest outcome in males of two subspecies of *Gallotia galloti* (Squamata: Lacertidae). *Behavioral ecology and sociobiology*, 72(3), 1-11.
- Brown, R. P., Hoskisson, P. A., Welton, J. H., & Baez, M. (2006). Geological history and within-island diversity: a debris avalanche and the Tenerife lizard *Gallotia galloti*. *Molecular Ecology*, 15(12), 3631-3640.
- Brown, R. P., Paterson, S., & Risse, J. (2016). Genomic signatures of historical allopatry and ecological divergence in an island lizard. *Genome biology and evolution*, 8(11), 3618-3626.
- Carracedo, J. C., Badiola, E. R., Guillou, H., Paterne, M., Scaillet, S., Torrado, F. P., Paris, R., Fra-Paleo, U. & Hansen, A. (2007). Eruptive and structural history of Teide

Volcano and rift zones of Tenerife, Canary Islands. *Geological Society of America Bulletin*, 119(9-10), 1027-1051.

Carracedo, J. C., Torrado, F. J. P., Ancochea, E., Meco, J., Hernán, F., Cubas, C. R., Casillas, R., Badiola E. R., Ahijado, A. (2002). Cenozoic volcanism II: the Canary Islands W. Gibbons, T. Moreno (Eds.), *The Geology of Spain*, The Geological Society, London, pp. 439-472.

Carretero, M. A. (2006): Reproductive cycles in Mediterranean lacertids: Plasticity and constraints. pp. 33-54, in: Corti, C.; Lo Cascio, P. & Biaggini M.(eds.) *Mainland and insular lacertid lizards: a Mediterranean perspective*. Firenze University Press. Florence

Carretero, M.A., Roig, J.M., Llorente, G.A. (2005): Variation in preferred body temperature in an oviparous population of *Lacerta (Zootoca) vivipara*. *Herpetological Journal*, 15: 51-55

Cox, C. L., & Cox, R. M. (2015). Evolutionary shifts in habitat aridity predict evaporative water loss across squamate reptiles. *Evolution*, 69(9), 2507-2516.

Del Arco, M., Pérez-de-Paz, P. L., Acebes, J. R., González-Mancebo, J. M., Reyes-Betancort, J. A., Bermejo, J. A., de-Armas, S & González-González, R. (2006). Bioclimatology and climatophilous vegetation of Tenerife (Canary Islands). In *Annales Botanici Fennici* (pp. 167-192). Finnish Zoological and Botanical Publishing Board.

Fariña, B., Navarro, M. L. R., López, M. R., & Moreno, J. E. (2011). Sobre la presencia de *Gallotia galloti* en el pico del volcán Teide (Tenerife, Islas Canarias). *Boletín de la Asociación Herpetológica Española*, (22), 46-51.

Ferreira, C. C., Santos, X., & Carretero, M. A. (2016). Does ecophysiology mediate reptile responses to fire regimes? Evidence from Iberian lizards. *PeerJ*, 4, e2107.

García-Muñoz, E. & Sillero, N. (2010). Two new types of noose for capturing herps. *Acta Herpetologica*, 5, 259-263.

Garcia-Porta, J., Irisarri, I., Kirchner, M., Rodríguez, A., Kirchhof, S., Brown, J. L., MacLeod, A., Turner, A. P., Ahmadzadeh, F., Albaladejo, G., Crnobrnja-Isailovic, J., De LA Riva, I., Fawzi, A., G., P., Göçmen, B., Harris, D. J., Jiménez-Robles, O., Joger, U., Jovanović Glavaš, O., Kariş, M, et al. & Valero, K. C. W. (2019). Environmental temperatures shape thermal physiology as well as diversification and genome-wide substitution rates in lizards. *Nature communications*, 10(1), 1-12.

Glazier, D.S. (2015). Is metabolic rate a universal 'pacemaker' for biological processes?

Biological Reviews, 90, 377–407

Gvozdík, L. & Castilla, A. M. (2001). A comparative study of preferred body temperatures and critical thermal tolerance limits among populations of *Zootoca vivipara* (Squamata: Lacertidae) along an altitudinal gradient in the Czech Republic. *Journal of Herpetology*, 35, 486–492.

Hammer, Ø., Harper, D. A., & Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia electronica*, 4(1), 9.

Hendry, A. P., Nosil, P., & Rieseberg, L. H. (2007). The speed of ecological speciation. *Functional Ecology*, 21(3), 455–464.

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G., & Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology: A Journal of the Royal Meteorological Society*, 25(15), 1965–1978.

Huey, R.B. & Bennett, A.F. (1987). Phylogenetic studies of coadaptation: preferred temperatures versus optimal performance temperatures of lizards. *Evolution*, 41, 1098–1115.

Kenner, R.A.; Ahmed, S.I. (1975). Measurements of electron transport activities in marine phytoplankton. *Marine Biology*, 33, 119–127.

Koziel, G.; S`khifa, A.; Kirchhof, S.; MacLeod, A.; Joger, U.; Sinervo, B.; Carretero, M. A.; Slimani, T. & Vences, M. (2021): Inferred preferred temperatures of lizards differ substantially among experimental methods. *Salamandra*, 57(2): 263–273.

Kunkel, G. (Ed.). (2012). *Biogeography and ecology in the Canary Islands* (Vol. 30). Springer Science & Business Media.

Lampinen, J.; Raitio, M.; Perala, A.; Oranen, H.; Harinen, R.-R. (2012) Microplate based pathlength correction method for photometric DNA quantification assay, in: Scientific, V.S.A.A.L.T.F. (Ed.).

Lleonart, J., Salat, J., & Torres, G. J. (2000). Removing allometric effects of body size in morphological analysis. *Journal of Theoretical Biology*, 205, 85–93

Megía-Palma, R.; Arregui, L.; Pozo, I.; Žagar, A.; Serén, N.; Carretero, M. A.; Merino, S. (2020): Geographic patterns of stress response in insular lizards reveal anthropogenic and climatic signatures. *Science of Total Environment*, 749, 141655.

- Molina-Borja, M., Padrón-Fumero, M. and Alfonso-Martín, T. (1997). Intrapopulation Variability in Morphology, Coloration and Body Size in Two Races of the Lacertid Lizard, *Gallotia galloti*. *Journal of Herpetology*, 31, 499-507.
- Qvarnström, A., Ålund, M., McFarlane, S. E., & Sirkiä, P. M. (2016). Climate adaptation and speciation: particular focus on reproductive barriers in *Ficedula* flycatchers. *Evolutionary applications*, 9(1), 119-134
- R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>
- Rivas-Martínez S. (1987). Mapa de series de vegetación de España 1:400000. Instituto Nacional para la Conservación de la Naturaleza (ICONA), Ser. Técnica, Madrid. 268 pgs
- Rundle, H. D., & Nosil, P. (2005). Ecological speciation. *Ecology letters*, 8(3), 336-352.
- Salvador, A. (2015). Lagarto tizón – *Gallotia galloti*. En: Enciclopedia Virtual de los Vertebrados Españoles. Salvador, A., Marco, A. (Eds.). Museo Nacional de Ciencias Naturales, Madrid. <http://www.vertebradosibericos.org>
- Schluter, D. (2009). Evidence for ecological speciation and its alternative. *Science*, 323(5915), 737-741.
- S'khifa, A., Koziel, G., Vences, M., Carretero, M. A., & Slimani, T. (2020). Ecophysiology of a lacertid community in the high Moroccan mountains suggests conservation guidelines. *Journal of Thermal Biology*, 94, 102743.
- Smadja, C. M., & Butlin, R. K. (2011). A framework for comparing processes of speciation in the presence of gene flow. *Molecular ecology*, 20(24), 5123-5140.
- Thorpe, R. S., & Richard, M. (2001). Evidence that ultraviolet markings are associated with patterns of molecular gene flow. *Proceedings of the National Academy of Sciences*, 98(7), 3929-3934.
- Thorpe, R. S., & Brown, R. P. (1989). Microgeographic variation in the colour pattern of the lizard *Gallotia galloti* within the island of Tenerife: distribution, pattern and hypothesis testing. *Biological Journal of the Linnean Society*, 38(4), 303-322
- Tosini, G., & Avery, R.A. (1993): Intraspecific variation in lizard thermoregulatory set points: a thermographic study in *Podarcis muralis*. *J. Therm. Biol.* 18: 19-23

Van Damme, R., Bauwens, D., Castilla, A.M., Verheyen, R.F. (1989): Altitudinal variation of thermal biology and running performance in the lizard *Podarcis tiliguerta*. *Oecologia (Berl.)* 80: 516-524.

Van Damme, R., Bauwens, D., Verheyen, R. (1990): Evolutionary rigidity of thermal physiology: the case of the cool temperate lizard *Lacerta vivipara*. *Oikos* 57: 61-67.

Veríssimo, C. V., & Carretero, M. A. (2009). Preferred temperatures of *Podarcis vaucheri* from Morocco: intraspecific variation and interspecific comparisons. *Amphibia-Reptilia*, 30(1), 17-23.

Wang, Y., Zeng, Z. G., Li, S. R., Bi, J. H. & Du, W. G. (2016). Low precipitation aggravates the impact of extreme high temperatures on lizard reproduction. *Oecologia*, 182, 961-971.

Zani, P. A. & Stein, S. J. (2018). Field and laboratory responses to drought in the common side-blotched lizards (*Uta stansburiana*). *Journal of Arid Environments*, 154, 15-23.

4.8. Supplementary materials

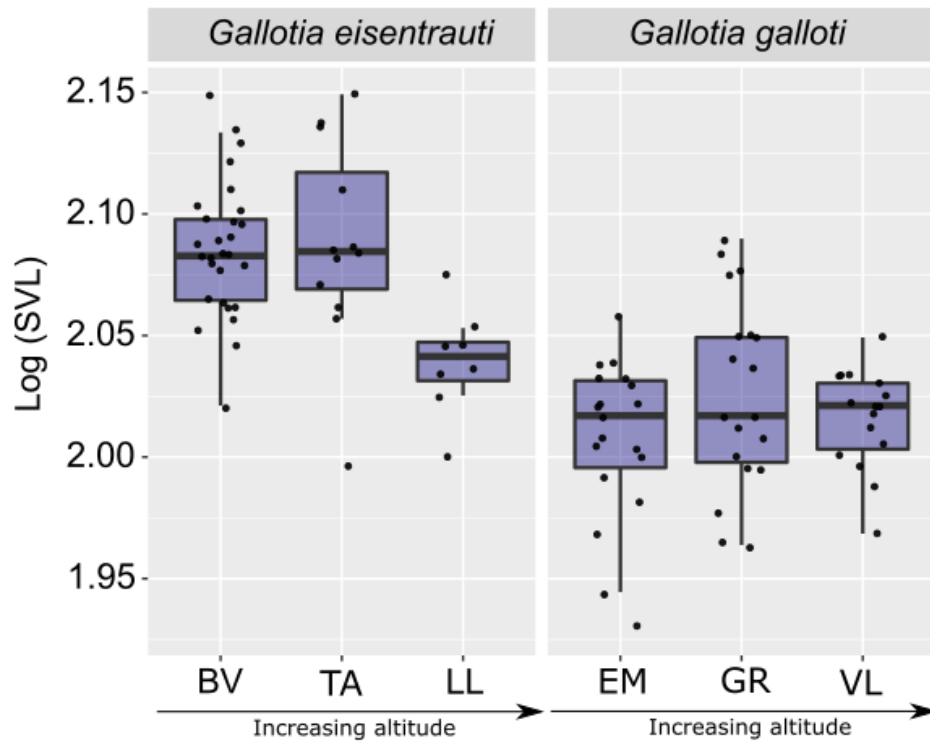


Figure S4.1. Box-and-jitter plot of log-transformed snout-vent length (SVL, a proxy of body size) for *Gallotia galloti eisentrauti* and *G. g. galloti*, collected at three different locations each (three altitudinal points).

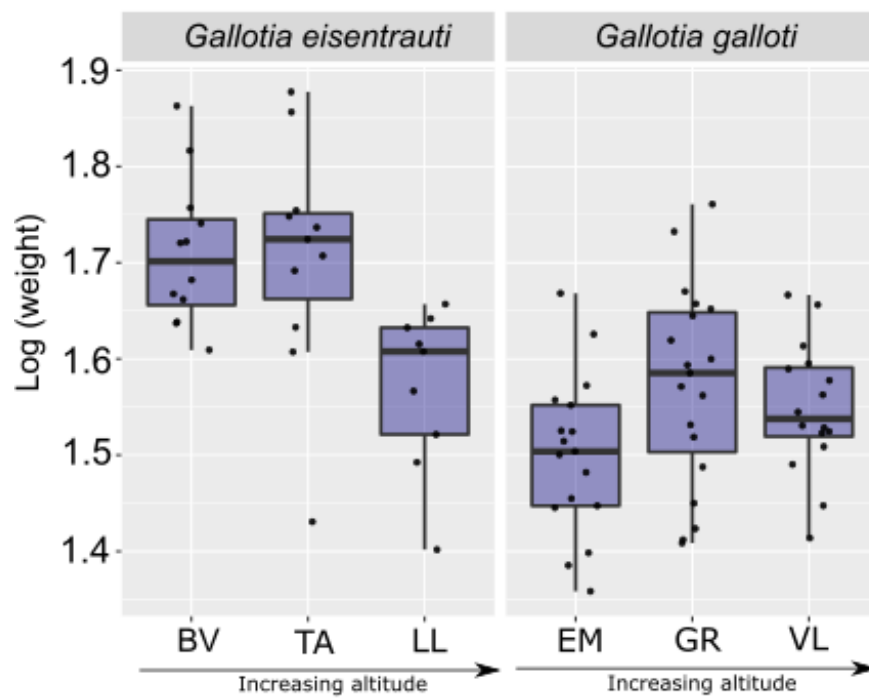


Figure S4.2. Box-and-jitter plot of log-transformed weight for *Gallotia galloti eisentrauti* and *G. g. galloti*, collected at three different locations each (three altitudinal points).

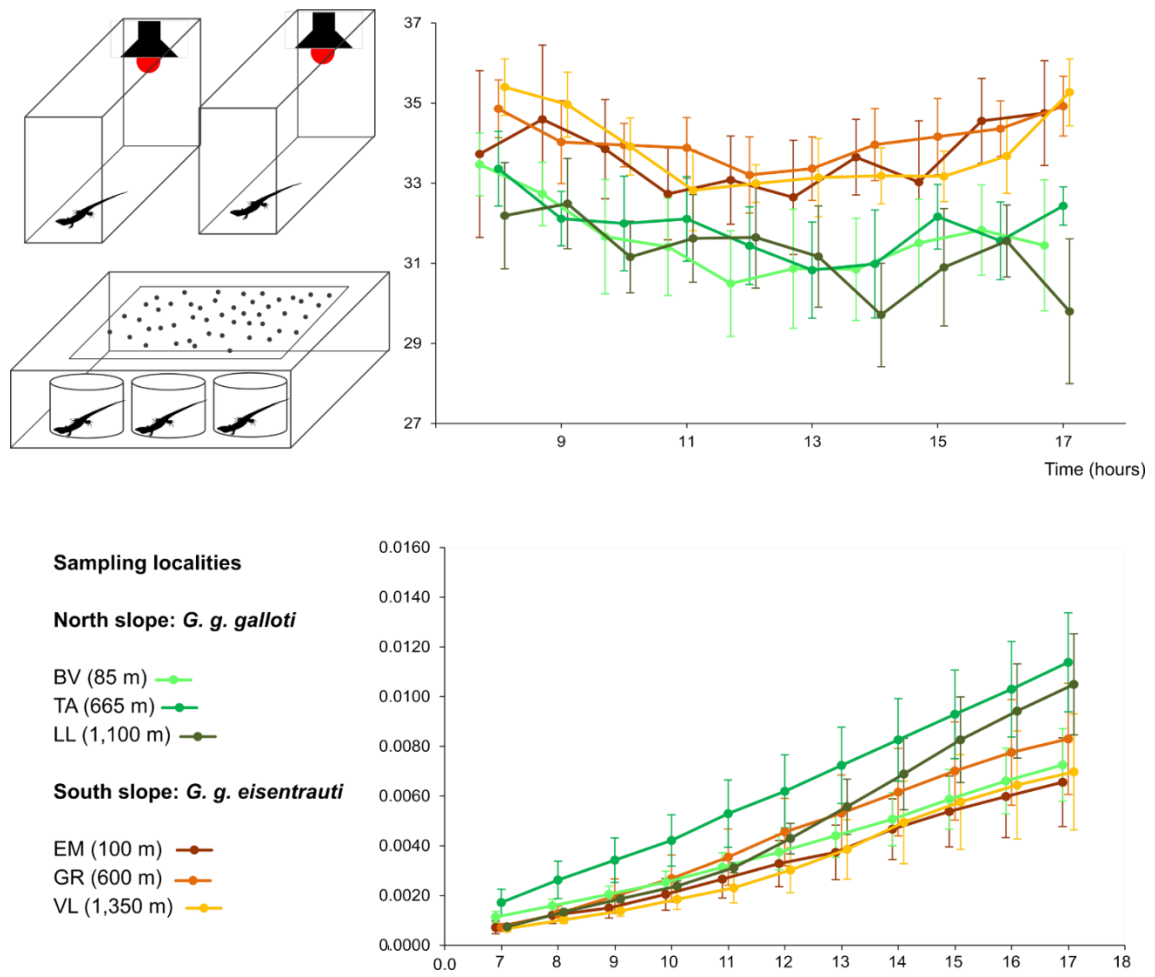


Figure S4.3. Plots with raw data for preferred temperatures (T_p , top) and accumulated water loss (EWLa, bottom) for *Gallotia galloti eisentrauti* and *G. g. galloti*, collected at three different locations each (three altitudinal points).

Table S4.1. Pairwise differences between all sampling localities for snout-vent length (SVL, proxy of body size). P-values were adjusted using the Benjamini-Hochberg method. Localities for *G. g. galloti*: EM, GR, VL. Localities for *G. g. eisentrauti*: BV, TA, LL.

Location1	Location2	P-value	P-value (adjustment)
EM	GR	0.069	0.103
EM	VL	0.441	0.473
GR	VL	0.327	0.386
EM	BV	< 0.001	< 0.001
GR	BV	< 0.001	< 0.001
VL	BV	< 0.001	< 0.001
EM	TA	< 0.001	< 0.001
GR	TA	< 0.001	< 0.001
VL	TA	< 0.001	< 0.001
BV	TA	0.824	0.824
EM	LL	0.019	0.032
GR	LL	0.335	0.386
VL	LL	0.089	0.122
BV	LL	< 0.001	0.001
TA	LL	0.001	0.002

Table S4.2. Pairwise differences between all sampling localities for weight. *P*-values were adjusted using the Benjamini-Hochberg method. Localities for *G. g. galloti*: EM, GR, VL. Localities for *G. g. eisentrauti*: BV, TA, LL.

Location 1	Location 2	<i>P</i> -value	<i>P</i> -value (adjustment)
EM	GR	0.020	0.034
EM	VL	0.138	0.188
GR	VL	0.431	0.539
EM	BV	< 0.001	< 0.001
GR	BV	< 0.001	< 0.001
VL	BV	< 0.001	< 0.001
EM	TA	< 0.001	< 0.001
GR	TA	< 0.001	0.001
VL	TA	< 0.001	< 0.001
BV	TA	0.926	0.959
EM	LL	0.066	0.099
GR	LL	0.959	0.959
VL	LL	0.554	0.640
BV	LL	0.001	0.002
TA	LL	0.001	0.003

Chapter V – General discussion

The main objective of the present thesis was to investigate how an ectotherm, the insular lizard *Gallotia galloti*, responds to the high environmental variability found in the island of Tenerife (part of the Canary Islands archipelago in the Atlantic), in particular variation associated with its extreme altitudinal distribution, and the multiple environmental challenges that come linked with it. Understanding this system required studying both the adaptive and plastic mechanisms that this insular lizard can exhibit across its distribution to cope with the environment, employing an approach based on the combination of multiple sources of data.

In general, we recovered relatively reduced variation in physiology, survivability and biochemistry in populations along a gradient of increased altitude. Among the few differences, there were in particular signs of reduced physiological stress (lower accumulation of carbonyl metabolites and longer relative telomere lengths) in highland populations relative to lowland. Sampled populations also differed in patterns of evaporative water loss, but these differences were seemingly not associated with altitude itself, but rather with local variation in body size and precipitation regimes.

We assembled for the first time a reference genome for a *Gallotia* species (Chapter III), and used this as a basis to examine the selection linked to altitudinal differences between populations at the extremes of the distribution. While for most of the genome we did not find outstanding signs of differentiation, exceptions were found in the vicinity of genes that are known in other organisms to be associated with the response to hypoxia, which has been a consistent constraint in other organisms facing high altitudinal stress.

We then expanded our approaches on physiology and metabolism to focus on the differences between two deep phylogenetic lineages of *G. galloti* that inhabit diverse climatic regimes in either side of Tenerife (Chapter IV): *G. g. galloti* inhabit the drier, southern side of the island, while *G. g. eisenrauti* can be found in the more humid northern side. Patterns of thermal and hydric regulation are in accordance with this distribution since, under similar experimental conditions, *G. g. eisenrauti* select for lower temperatures and have higher water loss. Our results suggest that *G. g. galloti* may be less constrained by the extreme environments at high altitude, and ultimately is likely to be better protected against longer term climatic changes.

5.1. Phenotypic plasticity helps to cope with drastic environmental changes

Results of ecophysiological tests did not reveal important changes in the way that different populations of *G. g. galloti* adjust their thermal physiology. Essentially these results suggest that, in their locations of origin (with very different environmental conditions in general), lizards have to thermoregulate by adjusting their activity patterns and microhabitat use to achieve very similar physiological optima. For example, lizards in higher altitudes should devote an increasing proportion of their activity to thermoregulation to achieve similar operating temperatures (or even not achieving their preferences, remaining thermally suboptimal) when compared to lowland lizards. Other examples of squamates that respond to colder temperatures in altitude through active thermoregulation include the viviparous lizard (*Zootoca vivipara*, Gvoždík, 2002) and the Atlas day gecko (*Quedenfeldtia trachyblepharus*, Bouazza et al., 2016). The top of the Teide experiences snowfall during the winter months, which should in principle make the habitat much less hospitable for an ectothermic lizard. So how do *G. galloti* living in the top of the Teide cope with the cold?

In general, high elevation populations of animals typically adopt life history strategies characterized by longer life spans, delayed maturity or dormancy which are commonly referred to as “slow life cycles” (Laiolo & Obeso, 2017). However, in our results we did not find evidence of different age structure between populations living at different altitudes, nor did we find indications that these lizards underwent brumation (which should have left a different trace of yearly skeletal growth profile comparing to low-altitude populations). Our biochemical assays also did not point to signals of higher physiological stress at altitude. Likely, local variation in microclimate as and trophic availability may have blurred geographic trends due to macroclimate. For instance, Fariña et al. (2011) reported that non-sulfurous vents near the volcano summit provide the lizards with the necessary source of heat they need to be active most of the year, even with snow cover. These also provide additional humidity and food sources (insects and plants) that should make them more resilient to the extreme harsh conditions at that altitude. Contrary to an absence of variation in thermal preferences, we found changes across populations in water loss rates. Differences in body size between populations may arise due to local variation in life-history and food availability, and subsequently can contribute to differences in water loss rates (smaller lizards have a higher surface-to-volume ratio and thus a higher rate of water loss across the skin).

The observation of overall similar thermal physiological optima across populations within the same lineage in this extreme gradient suggests that phenotypic plasticity, and not genetic variation, is involved with how they respond to their heterogeneous habitats, while differences between populations in hydric physiology suggest that local adaptation may be in play. To further disentangle plasticity from adaptation, translocations and acclimation of lizards to areas that are environmentally contrasting to their origin, followed by repeated testing, should provide additional evidence. A further indication that *G. galloti* apparently exhibits a more plasticity-mediated response for the tested traits is that our genomic scan results showed a relatively low genetic differentiation throughout the genome in the compared populations, with none of the identified outlier genomic regions containing genes that could be easily linked to a role in thermal or hydric ecology (although this interpretation should be taken with caution, see next section). Overall, altitudinal gradients provide good natural systems to conduct experiments to study how species respond to environmental change. However, it has to be taken into account that natural gradients are never straightforward or linear. As referenced in Chapter I, Tenerife has a relatively large variation in microclimate, vegetation type and topography, and many other abiotic factors also vary, either linearly or non-linearly with altitude (temperature, water availability, oxygen pressure, UV exposure) (Juan et al., 2000). Lizards were seemingly coping with this variability through thermoregulation, behaviourally selecting when, and where, to be active, which is likely to hinder evolutionary change (as suggested by the lack of clear signatures of adaptation). Our results could thus be seen as a demonstration of the Bogert effect in *G. g. galloti* (active thermal regulation delaying the need for adaptation). In the long term, however, this may not be enough to counteract the effects of climate change. This is demonstrated by our results comparing the two lineages (*G. g. galloti* and *G. g. eisentrauti*): lineages inhabiting areas with different microclimates show different physiological traits, which suggests that long term climatic change should lead to a restructuring of organismal physiology.

5.2. Selection on hypoxia-associated genes may explain adaptation to altitude

Our selection analyses based on whole-genome sequencing data pointed to two regions of the genome (on scaffolds 12 and 17) that were the clearest outliers, and these were located near genes with known links to hypoxia response in other organisms. The two most likely candidate genes were *BMPER* (bone morphogenetic protein-binding endothelial regulator, scaffold 12) and *ADRA1A* (adrenoceptor alpha-1A, scaffold 17), and both are involved in the formation of new blood vessels (angiogenesis) or the response to cardiac pressure overload (Heinke et al., 2008; Moretta et al., 2019; Eme et al., 2013). Expression studies have linked these genes directly or indirectly to hypoxia responses, indirectly in the case of *BMPER*, which regulates *BMP4* which is by itself upregulated in lung tissue during hypoxia. Interestingly, a gene close to *ADRA1A* is an additional potential candidate for adaptation to high altitude, *BNIP3L* (BCL2 interacting protein 3 like), since it is associated with mitochondrial autophagy, and subsequent reductions in mitochondrial volume (Bellot et al., 2009; Marinković & Novak 2021). These mitochondrial responses are known to occur in high-altitude adapted or non-adapted (acclimatized) humans, and the expression of this gene has been linked to hypoxia induced-factors. Several other regions with signatures of positive selection in the highland population were related with genes with known functions of interest such as *ENO1* (associated with resistance to hypoxia in pancreatic cancer cells; Wang et al., 2019) or *TPBG* (regulates angiogenic activity of pericytes; Spencer et al., 2019).

Among our outliers we did not detect any candidate that could be readily linked with other phenotypes that could be expected to be associated with altitudinal adaptation, such as high temperature, hydric stress or UV radiation. For example, it is plausible to expect that variation in genes related with DNA damage repair or melanin pigmentation could aid in responding to higher UV exposure in high altitudes (Li et al., 2018), but our analyses did not suggest this. It is possible that this general lack of signatures of selection throughout the genome is a consequence of two factors, either alone or combined. First, many of these adaptive traits can be associated with several selective sweeps of small intensity, which could make them hard to detect with our current dataset. Second, and as suggested by our physiology and metabolic tests, for many traits it is possible that phenotypic plasticity may be sufficient to overcome stress. Stronger signals of selection to counteract hypoxia may have arisen because low oxygen availability is one of the most difficult challenges that a lizard's organism can overcome with plasticity alone. In

fact, a widely known example of maladaptive plasticity in animals is precisely cardiac pathologies linked with non-adapted individuals to highland chronic hypoxia (Storz & Cheviron, 2021). These maladaptive plastic responses act in non-adapted individuals faced with occasional hypoxia stress, but a continuous exposure necessitates genetic compensation (Velotta & Cheviron, 2018). So, for these lizards, natural selection and phenotypic plasticity act in concert as major drivers of the success in high altitude colonization.

Despite finding high differences in allele frequency in variants near these hypoxia-associated genes, these were not fixed in highland populations, since highland alleles also appear in low frequencies in low-altitude populations. This suggests that standing genetic variation could be a very important mechanism for colonization of highland areas in this species. The environmental history of the Teide is likely to have been extremely dynamic, since it has been repeatedly exposed to glaciations and volcanic eruptions in the preceding hundreds of thousands of years. The maintenance of adaptive alleles in lowland populations could thus provide a means for repeated colonization of high altitude habitats. The occurrence of this scenario or a similar one is likely to have left signatures of genetic variation that could be uncovered with more detailed demographic reconstruction. Another way to gain further understanding on this system would be to collect samples along the altitudinal gradient to genotype variants to understand how allele frequencies shift along the gradient.

An additional and important missing piece of the puzzle is knowing specifically how these candidate genes could assist in adaptation. Functional validation of our results is necessary to fully understand how (and if) for example *BMPER*, *ADRA1A* and *BNIP3L* are actually contributing to the phenotype at high-altitudes. There are a few possibilities on how to do this, such as conducting RNA-sequencing studies to look into transcription differences in relevant tissues for hypoxia-associated phenotypes, such as lungs, blood or cardiac muscle (Todd et al., 2016). Current knowledge on the expression of *ADRA1A* and *BMPER* during induced hypoxia suggests that regulatory mutations in these genes could help in the mitigation of hypoxia through vascular cell proliferation (Li et al., 1995; Moreno-Miralles et al., 2011). In order to understand how these genes are regulated in hypoxia-adapted *G. galloti* analysing the expression of these candidate genes (and others involved in hypoxia signaling) through RNA-sequencing of individuals collected in their habitat of origin (lowland and highlands) is likely to be informative. However, more complex experimental designs involving reciprocally translocated lizards, or experimental testing of lizards under controlled atmospheres with different oxygen pressure could provide a better understanding on the expression of these genes. This is

particularly important since these genes could be involved in plastic maladaptive responses, thus needing genetic mutations to regulate their activity. Additionally, these gene expression assays should be accompanied by detailed phenotypic descriptions of the effects of hypoxia (e.g. Lui et al., 2015). Also, physiological measurement of cellular mitochondrial content and function could be a further source of data to consider (e.g. Lanza & Nair, 2010; McLaughlin et al., 2020), since this would allow to quantify the role of mitochondrial autophagy in this process, and hence potential contribution of *BNIP3L* to adaptation.

5.3. Knowing the evolutionary history of *G. galloti* is necessary to test functional hypotheses

Despite recent advances in our understanding on the functional biology of *Gallotia* lizards, both in the current thesis and other published works (Brown et al., 2016; Megía-Palma et al., 2020; Albaladejo et al., 2022), there is still limited knowledge on the underlying evolutionary and population history to help frame specific evolutionary hypothesis. A general phylogeographic hypothesis on the relationships between different species of *Gallotia* was generated by the work of Cox et al., (2010), based on four mitochondrial fragments. While this phylogeny is very well supported, additional genomic data using whole-genome sequencing or reduced representation sequencing (e.g. RAD-sequencing) would be informative to reconstruct not only the phylogenetic patterns but also each species' demographic history, patterns of genetic diversity and assess potential gene flow between species. It is however at finer phylogenetic scales that more gains could be made. For example, Brown et al. (2016) provided an initial overview on the patterns of genetic variation within *G. galloti* populations of Tenerife (both *G. g. galloti* and *G. g. eisentrauti*), but patterns of potential gene flow between populations inhabiting the different sides of the island (and likely belonging to different evolutionary lineages) were not explored. Patterns of variation are better known for *G. g. galloti*, with Brown et al. (2006) describing mtDNA substructure likely caused by an ancient debris avalanche of the Güimar valley.

Whole genome sequencing could also add valuable information to explore patterns of adaptation and genetic diversity at finer genomic resolution. This could even be performed using simple approaches based on sequencing single genomes from each species and using tools such as the pairwise sequentially Markovian coalescent (PSMC) (Mather et al., 2020). Given the likely dynamic nature of the habitats at the cone of the Teide during the last thousands of years, with a combination of glaciations and volcanism, combining these sources of data would provide very useful insights to understand how colonization of high altitude occurred, and why *G. g. eisentrauti* are seemingly unable to do it. If little or no gene flow between both lineages exists, competitive dynamics (mediated by past or contemporary climate) could still have promoted range exclusion. To understand if this effect occurs, behavioural, genetic and ecological studies on contact zones would be important to determine the extent of gene flow and the environmental context of the interaction between individuals of both lineages. In this context, our whole-genome reference assembly and annotation can

provide a valuable contribution for future studies, since it may provide a means to match functional testing with genomic features such as genes of known function or even the causal mutations leading to evolutionary novelty.

5.4. Concluding remarks

Conservation of island species is a matter of extreme importance, even for species such as *Gallotia galloti* that are common and widespread within their island of origin and thus considered as Least Concern for conservation. Biodiversity loss is a systemic worldwide problem, but islands are particularly affected by it due to their isolation, reduced area and relatively impoverished species richness, which makes biodiversity in these areas more vulnerable (Fernández-Palacios et al., 2021). A clear example of these vulnerabilities is given by the situation experienced less than 100 kilometres away, in another island of the Canary Islands archipelago, Gran Canaria. In this island an invasive exotic snake (*Lampropeltis californiae*) has been spreading quickly in the island's territory and annihilating endemic herpetofauna that are still classed as Least Concern. One of these is *Gallotia stehlini* for which, in places where this snake is present, population numbers decreased by 90% (Piquet & López-Darias, 2021). Other species of *Gallotia* are even in a more advanced state of decline: *G. simonyi* (El Hierro), *G. bravoana* (La Gomera) and *G. intermedia* (also in Tenerife), for which the causes of decline are mostly due to the introduction of alien predators combined with competition by smaller *Gallotia* species (Mateo et al., 2007). In examples such as these, understanding the capacity for a species to colonize a large range of environments may give us clues on how successful they may be in evading threats such as invasive species (for example, by inhabiting locations that a predator is not able to colonize). In general terms, understanding how this species adapts along an altitudinal gradient is useful to predict how this and other species may cope with ongoing climatic shifts, which in Tenerife are predicted to impact precisely areas of higher altitude (Martín et al., 2012). In this context, insights into how the different lineages of *G. galloti* adapted to either arid or humid habitats respond with environmental heterogeneity will be useful to track these changes). Our observation that physiological differences occur between lineages, and not between populations within the same lineage, suggests that adaptive responses evolve at a slow pace. This may induce lizard populations to collapse if environmental changes are too abrupt to be coped with phenotypic plasticity alone (García-Porta et al., 2019). Some bioindicators of stress recorded in the southernmost populations of *G. g. galloti* (Megía-Palma et al., 2020) suggest that they may be already undergoing such changes, although the situation will also be problematic if the humid and cold areas in the north that are favourable to *G. g. eisentrauti* shrink.

As mentioned above, multiple gaps in our knowledge on how our study species responds to environmental variation persist. These are mostly associated with understanding in detail the evolutionary history of the system, as well as describing the specific functional and molecular mechanisms that mediate adaptive responses and how they interact with the environment. Nevertheless, the work we conducted was an important and comprehensive first look into the functional responses to altitude in this unique ectothermal model system, one that is hoped to become an important reference for future studies on the adaptation of ectothermic vertebrates to large gradients of environmental variation.

5.5. References

- Albaladejo, G., Rodríguez, N., Rodríguez-Concepción, B., Nogales, M., & Vences, M. (2022). Limited ecophysiological variation in *Gallotia galloti* (Oudart, 1839) lizards across an elevational gradient of over 3,500 meters (Squamata: Lacertidae). *Herpetology Notes*, 15, 87-96.
- Bellot, G., Garcia-Medina, R., Gounon, P., Chiche, J., Roux, D., Pouysségur, J., & Mazure, N. M. (2009). Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Molecular and cellular biology*, 29(10), 2570-2581.
- Bouazza, A., Slimani, T., El Mouden, H., Blouin-Demers, G., & Lourdais, O. (2016). Thermal constraints and the influence of reproduction on thermoregulation in a high-altitude gecko (*Quedenfeldtia trachyblepharus*). *Journal of Zoology*, 300(1), 36-44.
- Brown, R. P., Hoskisson, P. A., Welton, J. H., & Baez, M. (2006). Geological history and within-island diversity: a debris avalanche and the Tenerife lizard *Gallotia galloti*. *Molecular Ecology*, 15(12), 3631-3640.
- Brown, R. P., Paterson, S., & Risse, J. (2016). Genomic signatures of historical allopatry and ecological divergence in an island lizard. *Genome Biology and Evolution*, 8(11), 3618-3626.
- Cox, S. C., Carranza, S., & Brown, R. P. (2010). Divergence times and colonization of the Canary Islands by *Gallotia* lizards. *Molecular Phylogenetics and Evolution*, 56(2), 747-757.
- Eme, J., Rhen, T., Tate, K. B., Gruchalla, K., Kohl, Z. F., Slay, C. E., & Crossley, D. A. (2013). Plasticity of cardiovascular function in snapping turtle embryos (*Chelydra serpentina*): chronic hypoxia alters autonomic regulation and gene expression. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 304(11), R966-R979.
- Fariña, B., Navarro, M. L. R., López, M. R., & Moreno, J. E. (2011). Sobre la presencia de *Gallotia galloti* en el pico del volcán Teide (Tenerife, Islas Canarias). *Boletín de la Asociación Herpetológica Española*, 22, 46-51.
- Fernández-Palacios, J. M., Kreft, H., Irl, S. D., Norder, S., Ah-Peng, C., Borges, P. A., Burns, K. C., Nascimento, L., Meyer, J.-Y., Montes & Drake, D. R. (2021). Scientists'

warning—The outstanding biodiversity of islands is in peril. *Global ecology and conservation*, 31, e01847.

Garcia-Porta, J.; Irisarri, I.; Kirchner, M.; Kirchhof, S.; Brown, J. L.; MacLeod, A.; Turner, A. P.; Ahmadzadeh, F.; Albadalejo, G.; Crnobrnja-Isailović, J.; de la Riva, I.; Fawzi, A.; Galán, P.; Göçmen, B.; Harris, D. J.; Jiménez-Robles, O.; Joger, U.; Jovanović-Glavaš, O.; Kariş, M.; Koziel, G.; Künzel, S.; Lyra, M.; Miles, D.; Nogales, M.J.; Oğuz, M. A.; Pafilis, P.; Rancilhac, L.; Rodríguez, N.; Rodríguez-Concepción, B.; Sanchez, E.; Salvi, D.; Slimani, T.; S'khifa, A.; Quasqaei, A.; Žagar, A.; Lemmon, A.; Lemmon, E.; Carretero, M. A.; Carranza, S.; Philippe, H.; Sinervo, B.; Müller, J.; Vences, M. & Wollemborg-Valero, K. C. (2019): Environmental temperatures shape thermal physiology as well as diversification and genome-wide substitution rates in lizards. *Nature Communications*, 10, 4077.

Gvoždík, L. (2002). To heat or to save time? Thermoregulation in the lizard *Zootoca vivipara* (Squamata: Lacertidae) in different thermal environments along an altitudinal gradient. *Canadian Journal of Zoology*, 80(3), 479-492.

Heinke, J., Wehofsits, L., Zhou, Q., Zoeller, C., Baar, K. M., Helbing, T., Laib, A., Augustin, H., Bode, C., Patterson, C. & Moser, M. (2008). BMPER is an endothelial cell regulator and controls bone morphogenetic protein-4–dependent angiogenesis. *Circulation research*, 103(8), 804-812.

Juan, C., Emerson, B. C., Oromí, P., & Hewitt, G. M. (2000). Colonization and diversification: towards a phylogeographic synthesis for the Canary Islands. *Trends in Ecology & Evolution*, 15(3), 104-109.

Laiolo, P., & Obeso, J. R. (2017). Life-history responses to the altitudinal gradient. In High mountain conservation in a changing world (pp. 253-283). Springer, Cham.

Lanza, I. R., & Nair, K. S. (2010). Mitochondrial metabolic function assessed in vivo and in vitro. *Current opinion in clinical nutrition and metabolic care*, 13(5), 511.

Li, H. T., Long, C. S., Rokosh, D. G., Honbo, N. Y., & Karliner, J. S. (1995). Chronic hypoxia differentially regulates $\alpha 1$ -adrenergic receptor subtype mRNAs and inhibits $\alpha 1$ -adrenergic receptor–stimulated cardiac hypertrophy and signaling. *Circulation*, 92(4), 918-925.

Li, J. T., Gao, Y. D., Xie, L., Deng, C., Shi, P., Guan, M. L., Huang, S., Ren, J. L., Wu, D. D., Ding, L., Huang, Z. Y., Nie, H., Humphreys, D. P., Hillis, D. M., Wang, W. Z. & Zhang, Y. P. (2018). Comparative genomic investigation of high-elevation adaptation in

ectothermic snakes. *Proceedings of the National Academy of Sciences*, 115(33), 8406-8411.

Lui, M. A., Mahalingam, S., Patel, P., Connaty, A. D., Ivy, C. M., Cheviron, Z. A., Storz, J. F., McClelland, G. B., & Scott, G. R. (2015). High-altitude ancestry and hypoxia acclimation have distinct effects on exercise capacity and muscle phenotype in deer mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 308(9), R779-R791

Marinković, M., & Novak, I. (2021). A brief overview of BNIP3L/NIX receptor-mediated mitophagy. *FEBS Open bio*, 11(12), 3230-3236.

Martín, J. L., Bethencourt, J., & Cuevas-Agulló, E. (2012). Assessment of global warming on the island of Tenerife, Canary Islands (Spain). Trends in minimum, maximum and mean temperatures since 1944. *Climatic Change*, 114(2), 343-355.

Mateo, J. A., Afonso, O. M. and Geniez, P. 2007. Los reptiles de Canarias, una nueva sinopsis puesta al día. *Boletín de la Asociación Herpetológica Española*, 2, 2-10.

Mather, N., Traves, S. M., & Ho, S. Y. (2020). A practical introduction to sequentially Markovian coalescent methods for estimating demographic history from genomic data. *Ecology and evolution*, 10(1), 579-589.

McLaughlin, K. L., Hagen, J. T., Coalson, H. S., Nelson, M. A., Kew, K. A., Wooten, A. R., & Fisher-Wellman, K. H. (2020). Novel approach to quantify mitochondrial content and intrinsic bioenergetic efficiency across organs. *Scientific reports*, 10(1), 1-15.

Megía-Palma, R., Arregui, L., Pozo, I., Žagar, A., Serén, N., Carretero, M. A., & Merino, S. (2020). Geographic patterns of stress in insular lizards reveal anthropogenic and climatic signatures. *Science of the Total Environment*, 749, 141655.

Moreno-Miralles, I., Ren, R., Moser, M., Hartnett, M. E., & Patterson, C. (2011). Bone Morphogenetic Protein Endothelial Cell Precursor-Derived Regulator Regulates Retinal Angiogenesis In Vivo in a Mouse Model of Oxygen-Induced Retinopathy. *Arteriosclerosis, thrombosis, and vascular biology*, 31(10), 2216-2222.

Moretta, D., Papamatheakis, D. G., Morris, D. P., Giri, P. C., Blood, Q., Murray, S., Ramzy, M., Romero, M., Vemulakonda, S., Lauw, S., Longo, L. D., Zhang, L. & Wilson, S. M. (2019). Long-term high-altitude hypoxia and alpha adrenoceptor-dependent pulmonary arterial contractions in fetal and adult sheep. *Frontiers in Physiology*, 1032.

- Piquet, J. C., & López-Darias, M. (2021). Invasive snake causes massive reduction of all endemic herpetofauna on Gran Canaria. *Proceedings of the Royal Society B*, 288(1964), 20211939.
- Spencer, H. L., Jover, E., Cathery, W., Avolio, E., Rodriguez-Arabaolaza, I., Thomas, A. C., Alvino, V. V., Sala-Newby, G., Dang, Z., Fagnano, M., Reni, C., Rowlinson, J., Vono, R., Spinetti, G., Beltrami, A. P., Gargioli, C., Caporali, A., Angelini, G., & Madeddu, P. (2019). Role of TPBG (trophoblast glycoprotein) antigen in human pericyte migratory and angiogenic activity. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 39(6), 1113-1124.
- Storz, J. F., & Cheviron, Z. A. (2021). Physiological genomics of adaptation to high-altitude hypoxia. *Annual review of animal biosciences*, 9, 149.
- Todd, E. V., Black, M. A., & Gemmell, N. J. (2016). The power and promise of RNA-seq in ecology and evolution. *Molecular ecology*, 25(6), 1224-1241.
- Velotta, J. P., & Cheviron, Z. A. (2018). Remodeling ancestral phenotypic plasticity in local adaptation: A new framework to explore the role of genetic compensation in the evolution of homeostasis. *Integrative and comparative biology*, 58(6), 1098-1110.
- Wang, L., Bi, R., Yin, H., Liu, H., & Li, L. (2019). ENO1 silencing impairs hypoxia-induced gemcitabine chemoresistance associated with redox modulation in pancreatic cancer cells. *American Journal of Translational Research*, 11(7), 4470.