Reptiles under the sun: using lacertid lizards to study thermal and water ecology in ectotherms

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Ai miei genitori e ai miei nonni

Nota prévia

Na elaboração desta tese, e nos termos do número 2 do Artigo 4o do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do D.L. 74/2006, de 24 de Março, com a nova redação introduzida pelo D.L. 230/2009, de 14 de Setembro, foi efetuado o aproveitamento total de um conjunto coerente de trabalhos de investigação já publicados ou submetidos para publicação em revistas internacionais indexadas e com arbitragem científica, os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua conceção, na obtenção e análise de dados, e discussão de resultados, bem como na elaboração da sua forma publicada.

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Acknowledgments

The present work is the waypoint that marks the last four years of my voyage in Science as well as in life. I came to the conclusion that doing a doctorate is like searching for a constant balance between a personal and solitary journey of growing on the one hand and the need to reach out to other people to get help and improve on the other side. As a result, I profoundly changed my attitude during the last four years. I was confronted with living in a new country, learnt two other languages, met many new people and lost some old ones. I'm proud to say that I grew not only as a scientist but most importantly as a person. Hence, even though the work I present and discuss in the next pages is mine, it would not exist in this form without the collaboration, the input, and the help of several people and institutions.

Miguel Angel Carretero and I met during a congress in Slovenia in 2013. Later on, I expressed to him my interest in pursuing a PhD. Since the beginning, he was open-minded and interested in my ideas, and in 2015 I finally moved to work together. It has not always been easy between us. We are both stubborn and defend our positions fiercely. I'm glad about that. I would not have grown so much without open and, sometimes, dashing confrontations. Gracias Miguel por tu apoyo en estos años, por aguantar mi cabezonería y sangre a veces demasiado caliente, y por entender y aceptar mi situación personal de gitano entre varios países.

The FCT, Fundação para a Ciência e a Tecnologia believed in my project and financed it. Without its financial support, this thesis would have not even started. Similarly, CIBIO hosted me for four years and gave me the occasions to meet many other researchers and to a place to follow courses and seminars. Finally, the ICNF, Instituto da Conservação da Natureza e das Florestas, and especially Marina Sequeira, provided me with all the permits I needed to carry on my research. To all these institutions, obrigado.

While developing my doctorate during the last four years, I met many people from different countries. Some of them contributed substantially to some of my work, like Frederico Barroso. We shared many adventures, and I rapidly became my best friend in Portugal and an example of dedication and hard work. Other people were kind enough to invest some of their time teaching me new concepts and techniques, like Angelica Crottini and Walter Cocca. Finally, I shared little as well as big moments of friendship with people like Lucia Perez and Michael Jowers. The physical places in which I have lived for enough time become particularly important for me. The rhythms, the landscapes,

the people, all fade into memories that often came back, making me remember where I have

been and to what I bound. I'll always have sweet memories for many places in Portugal, but especially for Vila do Conde, in which I lived –and often worked– for almost four years.

In Vila, I met two beings that would turn to be essential for me to endure the marathon that the present work has represented successfully. The first was a lovely cat called Memeo initially –but now that she lives in Spain she is just Meme. People that live alone and are lucky enough to share the company of an affectionate cat will understand my feelings without further explaining.

And finally, I met Raquel. I will not say that without her I would not have finished my PhD. Instead, if I had dedicated four years of my life only to research, in a monk-style way of life, I would have perhaps produced more science. But a realized that life is not just about working hard, doing science and rehearse.

Hemos pasado de todos en estos últimos casi cuatro años juntos y, no obstante no siempre hayamos comido perdices, lo haría todo otra vez. Gracias por haber entrado en mi vida aquella noche de fiesta y, sobre todo, por haberte quedado desde entonces.

Resumo

Desde há vários séculos que os répteis têm vindo a ser considerados como animais "de sangue frio" tendo até mesmo Linnaeus, no seu *Systema Naturae,* colocado estes em conjunto com os anfíbios. No entanto, desde a década de 40 do século passado, alguns investigadores começaram a medir a temperatura corporal de répteis no campo. Por sua vez, estas investigações levaram à descoberta de que os répteis possuem a capacidade de manter a sua temperatura corporal acima da temperatura do ar, conseguindo até manter temperaturas corporais constantes durante uma quantidade considerável de tempo, principalmente através de termorregulação comportamental. Nas seguintes décadas, foram publicados milhares de artigos sobre a ecologia térmica de répteis. Nos últimos anos, com o aumento do interesse mundial no efeito potencialmente adverso das mudanças climáticas na biodiversidade, estudos sobre ecologia térmica de répteis têm vindo a ganhar um valor científico crescente, tomando por exemplo estudos que demonstraram que até um quinto de todas as populações de répteis podem vir a ser extintas até o final do século.

Em contrapartida, o equilíbrio hídrico dos répteis recebeu comparativamente menos atenção. Na verdade, a água tem sido muitas vezes associada a questões de anfíbios, enquanto que a temperatura é associada mais aos répteis. No entanto, nos últimos anos, vários trabalhos têm começado a explorar os potenciais efeitos adversos da escassez de água, desidratação e regimes de chuvas na biodiversidade de répteis, bem como na sua distribuição e atividade. Além disso, prevê-se que as alterações climáticas aumentem a frequência das secas e das vagas de calor, especialmente em algumas zonas como a bacia mediterrânica. Assim sendo, chegou o memento de adotar uma abordagem mais holística sobre ecologia dos répteis, onde a ecologia térmica se entrelaça o máximo possível com outros aspectos como a fisiologia e história de vida. Na verdade, apenas um quadro de investigação mais abrangente poderá permitir-nos obter uma visão mais aprofundada sobre a biologia dos répteis e assim desenvolver ferramentas preditivas e de gestão mais poderosas para compreender se e como este grupo de animais reagirá às futuras condições climáticas e ambientais bem como aos noves desafios que poderão enfrentar.

No presente trabalho, descrevo e discuto os resultados de seis investigações destinadas a reforçar a compreensão da ecologia térmica dos répteis, do balanço hídrico e das interações potenciais entre esses dois aspectos fundamentais da biologia reptiliana.

A temperatura corporal dos répteis pode ser recolhida de várias maneiras. Um dos métodos mais comuns é usar um termopar para medir a temperatura cloacal. No

entanto, hoje em dia novas ferramentas, como câmeras infravermelhas portáteis, têm vindo a melhorar significativamente vários setores, desde a indústria e da defesa à medicina diagnóstica. A investigação sobre répteis pode também beneficiar da incorporação de dados de infravermelho, que permitem novas formas de estudar ecologia térmica. Um primeiro grande desafio é provar que novas ferramentas são fiáveis e comparáveis com as mais comuns e difundidas técnicas. Aqui, o primeiro passo foi demonstrar a relação robusta entre as leituras de temperatura cloacais e termográficas. De seguida, a investigação moveu-se para o campo para investigar, usando a imagem infravermelha, um aspecto mal estudado do comportamento anti-predador dos lagartos. Na verdade, quando os lagartos se escondem de um predador, é provável que sua temperatura corporal diminua com o tempo. Tal diminuição da temperatura corporal pode representar um custo em termos de aptidão (*fitness*) se os lagartos são forçados a perder tempo a termorregular, após cada tentativa de predação, perdendo assim a oportunidade de se alimentarem ou de acasalarem. Neste trabalho, foi quantificada pela primeira vez a diminuição da temperatura corporal que os lagartos sofrem ao se esconderem de um predador.

Outra linha de investigação deste projeto, focou-se na ecologia térmica, balanço hídrico e desempenho de espécies intimamente relacionadas que coexistem. Estudos sobre vários grupos de organismos indicam que em casos de coexistência entre espécies estreitamente relacionadas, estas reduzem a potencial concorrência segregando-se no tempo ou espaço ou sofrendo alterações suficientes (deslocamento de caráter) para reduzir a sobreposição dos seus nichos. Em estudos de ecologia térmica, dependendo do caso, espécies intimamente relacionadas mostram conservatividade ou variabilidade nas preferências térmicas. Aqui foram encontradas pequenas diferenças nas preferências térmicas quando comparadas duas espécies-irmãs, *P. bocagei* e *P. guadarramae* provenientes da mesma área. Estas espécies não mostraram diferenças no desempenho motor, no entanto, apresentaram maior variabilidade na resistência ao balanço hídrico.

Finalmente, os dois últimos estudos incidiram sobre a potencial interação entre ecologia térmica e equilíbrio hídrico, bem como sobre sua variabilidade inerente entre as populações. Na primeira experiência, a desidratação prejudicou a termorregulação em quatro espécies de lagartos. De facto, os lagartos desidratados reduziram a sua temperatura corporal e retiravam-se com maior frequência para dentro dos refúgios fornecidos. No segundo estudo, no lagarto *Psammodromus algirus*, o estudo de campo encontrou pouca variação na temperatura corporal entre as populações. No entanto, as temperaturas corporais do lagarto mostraram flexibilidade diária e sazonal.

Contrariamente, a resistência à perda de água aumentou com a altitude, mas não mostrou variabilidade sazonal.

Em suma, as investigações apresentadas nesta tese permitiram (1) validar o uso de câmeras infravermelhas em estudos de ecologia térmica em lagartos, demonstrando a relação robusta entre a temperatura superficial e a temperatura interna; (2) investigar no terreno o comportamento evasivo dos lagartos quantificando pela primeira vez a diminuição da temperatura corporal que se segue após uma tentativa de predação; (3) quantificar a diferença na temperatura corporal preferida e resistência à perda de água em espécies intimamente relacionadas vivendo em simpatria; (4) comparar o desempenho motor de tais espécies estreitamente relacionadas e avaliar a sua sensibilidade térmica; (5) compreender como e em que medida a desidratação prejudica a termorregulação e o uso do espaço em pequenos lagartos e (6) avaliar se as preferências térmicas e a resistência à desidratação podem mostrar variação entre as populações através de um gradiente altitudinal.

A contribuição dos trabalhos apresentados tem o potencial para ter implicações a nível metodológico e ecológico. Novas tecnologias e ferramentas, como imagens infravermelhas, permitirão responder a questões fisiológicas e comportamentais que não podem ser abordadas com métodos mais tradicionais. O comportamento de evasão dos lagartos fornece apenas um exemplo, aqui investigado. Da mesma forma, o facto de que espécies intimamente relacionadas poderem coexistir apesar de pouca diferenciação, tanto a nível fisiológica como a nível do organismo com um todo, padece de mais estudos. Finalmente, vários estudos, incluindo os dois últimos aqui apresentados, apontam para a possível interação adversa entre a termorregulação e o balanço hídrico em diversas espécies de lagartos. Num futuro breve, será necessário investigar ainda mais essa interação, especialmente à luz das pressões climáticas e ambientais que a humanidade está a colocar por todo o mundo e, em particular, aos lagartos.

Palavras chave: Ecologia térmica, temperatura corporal preferida, balanço hídrico, desidratação, tecnologia infravermelha, Lacertidae; *Timon*, *Psammodromus*, *Podarcis*

Summary

Reptiles have been considered “cold-blooded” for centuries, and even Linnaeus in his *Systema Naturae* placed them together with amphibians. However, from the ’40 of the previous century, a few researchers started to measure reptiles body temperature in the field. They soon discovered that reptiles can maintain their body temperature warmer than air temperature and to keep it constant over a considerable amount of time mainly by behavioural thermoregulation. In the following decades, thousands of articles investigated reptile thermal ecology. In recent years, along with the worldwide raising interest in the potential adverse effect of climate change on biodiversity, studies on reptile thermal ecology are gaining increasing scientific value, for example by showing that up to one-fifth of all reptile populations may go extinct by the end of the century.

By contrast, water balance in reptiles has received comparatively less attention. Indeed, water has been often considered an amphibian’s issue, and temperature a reptile’s one. However, in recent years, several works are exploring the potential adverse effects of water shortage, dehydration, and rainfall regimes on reptile biodiversity, distribution and activity. Furthermore, climate change is predicted to increase the frequency of droughts and heatwaves, especially in some areas like the Mediterranean basin. Thus, the time has come for research on reptile thermal ecology to intertwine as much as possible with other aspects of their ecology, physiology and life-history. Indeed, only a more comprehensive research framework may allow us to gain deeper insight on reptile biology, and to develop more powerful predictive and managing tools to understand if and how this group of animals will react to the future climatic and environmental challenges they face.

In the present work, I describe and discuss the results of six investigations aimed at reinforcing our understanding of reptile thermal ecology, water balance, and the potential interactions between these two fundamental aspects of reptilian biology.

The body temperature of reptiles can be collected in many ways. One of the most common methods is to use a thermocouple to measure the cloacal temperature. Nowadays new tools, like portable infrared cameras, are significantly improving several sectors, from industry and defence to diagnostic medicine. Research on reptiles may benefit from incorporating infrared data, that allows for new and alternative ways to study thermal ecology. A first big challenge is to prove that new tools are reliable and comparable with the most common and widespread ones. Here, the first step has been to demonstrate the robust relationship between cloacal and infrared readings. The

investigation then moved in the field to investigate, using infrared imaging, a poorly studied aspect of antipredator behavior of lizards. Indeed, when lizards hide from a predator, it is likely that their body temperature decreases with time. Such a decrease in body temperature may represent a cost in terms of fitness if lizards are forced to waste time thermoregulating after each predation attempt, losing the opportunity to feed or mate. In this work, it has been quantified for the first time the decrease in body temperature that lizards suffer while hiding from the predator.

Another line of investigation in this project focused on the thermal ecology, water balance and performance of closely related species that coexist. Research on several groups of organisms indicates that coexisting closely related species reduce potential competition by segregating in time or space or by changing enough (character displacement) to reduce niche overlap. In thermal ecology studies, depending on the case, closely related species both show conservativeness or variability in thermal preferences. Here little differences in thermal preferences were found comparing two sister species, *P. bocagei* and *P. guadarramae* collected in the same area. Similarly, they do not differ in running performance. However, these species showed more variability in their resistance to water balance.

Finally, the last two studies focused on the potential interaction between thermal ecology and water balance, as well as on their inherent variability among populations. In the first experience, dehydration impaired thermoregulation in four lizard species. Indeed, dehydrated lizards reduced their body temperature and retreated more often inside the refuges. In the second study, on the lizard *Psammodromus algirus*, the field survey found little variation in body temperature across populations. However, lizard body temperatures showed daily and seasonal flexibility. On the contrary, resistance to water loss increased with altitude but showed no seasonal variability.

In summary, the investigations presented in this thesis allowed to (1) validated the use of Infrared cameras in thermal ecology studies on lizards, by demonstrating the robust relationship between surface and core temperature; (2) investigated lizard hiding behavior in the field and quantified for the first time the decrease in body temperature that follows after a predation attempt; (3) quantified the difference in preferred body temperature and resistance to water loss in closely related species living in sympatry; (4) compare the running performance of such closely related species and assess their thermal sensitivity; (5) understand how and to what extent dehydration impairs thermoregulation and space use in small lizards and (6) evaluate if thermal preferences and resistance to dehydration may show variation among populations across an altitudinal gradient.

The contribution of the presented works may have, potentially, methodological and ecological implications. New technologies and tools, like infrared imaging, will allow answering physiological and behavioural questions that cannot be tackled with more traditional methods. The lizard hiding behavior provides an example, investigated here. Similarly, the fact that closely related species may coexist despite little physiological and whole-organisms differentiation calls for more studies on this subject. Finally, several studies, including the last two presented here, are pointing out the potential adverse interaction between thermoregulation and water balance in several lizard species. In the following years, it will be necessary to investigate further such an interplay, especially in the light of the climatic and environmental pressures that humankind is posing worldwide on lizards.

Keywords: Thermal ecology, Preferred body temperature, Water balance, Dehydration, Infrared technology, Lacertidae; *Timon*, *Psammodromus*, *Podarcis*

Index

List of Figures

Note. In the present thesis, the numeration starts with the chapter number followed by the figure or table number. In the six published or submitted papers, the original numeration has been changed to avoid confusion.

List of Tables

List of Abbreviations

EWL	Evaporative Water Loss
IRT	Infrared Thermal Imaging
TEWL	Total Evaporative Water loss
SVL	Snout-Vent length
$\mathbf{T_o}$	Optimal temperature for a physiological function
$\mathbf{T_{pref}}$	Preferred body temperature

Chapter 1: Introduction

“I am not apt to follow blindly the lead of other men.”

— Charles Darwin (1887)

1.1. Temperature?

Temperature is so fundamental for life that not surprisingly is one of the most frequent topics in our daily business. Meteorologists strive to continuously forecast the weather—including temperature—for the next following days, and people based much of their daily and seasonal activity and temperature fluctuations. However, it is often somewhat painful for non-scientists people to precisely state what temperature is. Even within the scientific paddock —apart for physicists— several terms are commonly used as synonyms for temperature. In my experience, the most typical source of confusion comes from the term *temperature, heat* and *energy*.

Temperature can be defined as the physical property of a system which indicates the flow direction of *energy*. When two systems have different temperatures, energy will flow from the warmest to the coldest in the form of *heat*, until the temperature of the two systems equilibrates (Schroeder 1999).

Even though the definition of temperature, heat and energy might seem trivial or academic, it serves here as a base reference around which the hypotheses, experiments and conclusions are built in this thesis. To give a paradoxical example, the unit of temperature in the International System of Units is the *kelvin* (lower-case), with symbol K. However, even though the present thesis follows the IS in any other aspects, I never use the kelvin here. The contradiction may appear relevant, considering that in the final version of this thesis the word *temperature* appears 992 times! Far from being a personal whim, I use here the Celsius scale for the straightforward reason that virtually all the literature relevant for this thesis is published using the Celsius scale as a reference instead of the kelvin scale. Even though some seminal papers on thermal ecology were published using the Fahrenheit scale, the same authors switched in a few years to the Celsius scale (Cowles and Bogert 1944; Bogert 1949). Why the Celsius scale is so widespread at the expense of the kelvin I guess has much to do with the values that the two scales have at biologically relevant processes. For example, it is probably easier to visualize a change from 20 to 21 °C than the same transition from 293 to 294 K.

Temperature, in conjunction with water (discussed later), does not only permeate virtually every aspect of life, but also defines the very limits within which life can support itself. For example, archaea can grow at temperatures as high as 122 °C, while the upper limit for eukaryotes is around ~60 °C. Similarly, while some plants and invertebrates can endure temperatures as low as -70 °C, the lower limit to sustain life cycle in multicellular organisms is around ~-2 °C (Clarke 2014). All species used in this thesis as a model are lacertid lizards. Hence temperature affects their physiology, growth, reproduction and locomotion usually in the range comprised between ~0 and 40 °C (Angilletta 2009). Even though most of the energy on our planet comes ultimately from the sun, at some point during animal evolution, few species evolved the ability to increase their metabolism high enough to produce heat from the chemical bonds contained in the food. Even though they represent a tiny fraction of all animal species, birds and mammals represent a rupture with the rest of animal species, and their physiological differences are so fundamental to the point of determining why a 2 g hummingbird has to feed constantly while a small lacertid lizard of 2 g can feed once a month.

1.2 Ectotherms versus Endotherms

With the term *ectotherm* are defined all those species—the vast majority of animal species—that are unable to raise their body temperature above air temperature by producing metabolic heat. By contrast, the term *endotherm* applies to those species—mostly birds and mammals—that can increase their body temperature and keep it constant through metabolism (Fig. 1.1. See the next section for more terminology).

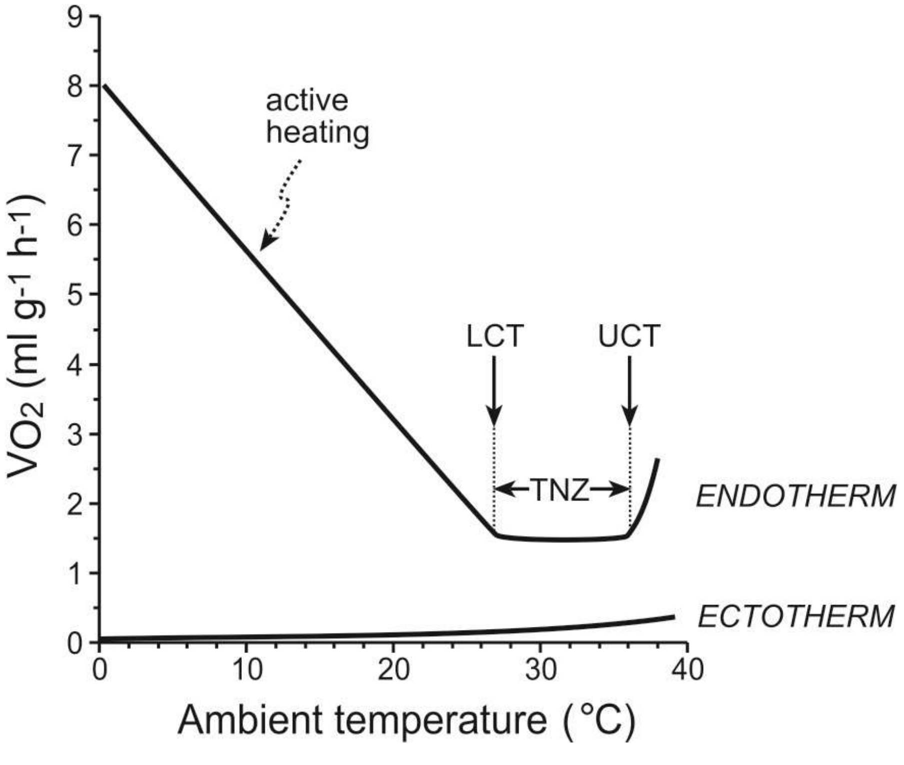

Fig. 1.2.1. Comparison of the oxygen consumption as a function of environmental temperature by similar size endotherms (top curve) and ectotherms (bottom curve). The metabolic rate of ectotherms follows Arrehenius (Q_{10}) effects. Instead, the metabolic rate of endotherms is constant within the thermal neutral zone (TNZ), increases with decreasing temperatures below the lower critical temperature (LCT) due to the energy cost of heat production, and increases with increasing temperatures above the upper critical temperature (UCT), first as a result of active cooling and then when tissue temperatures begin to rise as a result of Q_{10} effects. Adapted from Hiebert and Noveral (2007).

Apart from a higher—and energetically inefficient—metabolism, endotherms also evolved effective traits to reduce heat dissipation into the environment. From reptilian scales, birds evolved the feather, while mammals evolved the hair (but feathers have been found in dinosaurs and flying reptiles as well: Xu et al. 2003; Yang et al. 2019). Even though the distinction between ectotherms and endotherms may appear neat, many exceptions blur the edges of the two categories. For example, many insect species can raise their body temperature by intense muscle contraction in preparation for flight (Bartholomew and Casey 1977). Similarly, several species of scombroid fishes evolved various anatomical and physiological features that enable them to sustain a much higher metabolic rate than most fish (Korsmeyer and Dewar 2001). The leatherback turtle is one of the reptile species with the broadest distribution range across the world and can venture in polar regions due to what is called *gigantothermy*, that is its ability to raise its core temperate through muscle action and retain heat thanks to a thick layer of brown tissue (Paladino et al. 1990). It is thus clear that the ectotherm/endotherm dichotomy is overly simplistic, with many species from very small like moths to very large like marine turtles able to maintain their body temperature higher than the surrounding medium. Even dinosaurs, considered for a long time as lumbering and lazy ectotherms, have been reconsidered as possibly endotherms (Bakker 1972). Recent investigations suggest that dinosaurs' metabolism was probably high enough to be comparable to that of leatherback turtles (ectotherms) or echidnas (endotherms) and classify them as *mesotherms* (Grady et al. 2014).

When I was an undergraduate student following the Physiology classes, I remember the professor highlighting birds and mammals' supposed superiority due to their ability to generate and retain enough metabolic heat to be virtually independent of an external source of energy, apart from food. Still today, terms like *higher vertebrate* (birds and mammals) and *lower vertebrate* (fish, amphibians and non-avian reptiles) are not so infrequent. Hence, ectotherms are often considered inferior because they never evolved such an advantageous trait like producing metabolic heat. Even Linnaeus (1758) considered cold-blooded animals as “foul and loathsome” and placed amphibians and reptiles together while commenting “the Lord has created so few of them”. The view of ectotherms as slow and clumsy animals was so rooted in society that it took a movie (Jurassic Park, 1993) to shift the mind of non-scientists and force people to see ectotherms (and mesotherms) as dynamic and active animals.

What are the advantages of being an endotherm? And, put differently, is ectothermy an inferior condition of life?

As far as I am aware of, Pough (1980) was the first one in analyzing and defending the advantages of ectothermy and pointing it out as an adaptation instead of a primitive feature. Ectotherm metabolism is low, and their mitochondria do not produce enough heat to raise their body temperature significantly. Indeed, ectotherms generally gain heat from the sun, either directly by exposing themselves to sun's radiation, or by absorbing heat from the environment, like placing themselves on a hot rock. Furthermore, ectotherms are small in comparison with endotherms. Over 80% of reptile species weight less than 20 g (Pough 1980). Because metabolism is mainly determined by body size and increases non-linearly with temperature (Hawkins 1995; Gillooly et al. 2001), ectotherms spend most of their life at low metabolic rates and are exceptionally efficient in energy conversion, especially during early life stages (Wieser 1985). Furthermore, with some notable exceptions (like the mentioned tunas and marine turtles), a high level of aerobic activity is generally not possible, and bursts of anaerobic action is much more common. Instead, endotherms can sustain prolonged periods of aerobic activity, can be active at any hour of the day, any time of the year and virtually in every corner of the world.

Endothermy, however, comes at a constant price. To sustain the inefficient conversion of chemical energy (i.e. food) to heat, mitochondria need a continuous energy supply. Endotherms need to eat regularly to compensate the heat dissipation. For example, the oxygen consumption at ~20°C of the Italian wall lizard *Podarcis siculus* is 0.80 $mLg^{-1}h^{-1}$, while at the same temperature the Eurasian pygmy shrew *Sorex minutus* consumes 28.7 $mLg^{-1}h^{-1}$, that is almost 36 times higher (Cragg 1978; Sparti 1992). Even those endotherms that undergo prolonged periods of reduced activity, often referred to as hibernation, like several mammals and a few bird species, still often maintain relatively high body temperature and metabolic rates, if compared to ectotherms' (Gillooly et al. 2001; Geiser 2004).

1.3 Definition of some thermal labels

In the previous section, I discussed the physiological differences that differentiate ectotherms and endotherms are and how such differences determine every aspect of their life. However, during the last century of thermal ecology studies, researchers have defined many physiological and ecological categories in an attempt to classify animal species. Even though categories and labels have the benefit of helping the human mind to put order and organize, they also have the cons of creating the opposite result, that is to create chaos if labels are not applied most rigorously.

Poikilothermy and *homoiothermy* refer to fluctuating versus constant body temperatures, respectively. Often the two terms are used as a synonym of ectotherms and endotherms, while it is clear today that a thermoregulating lizard might keep a stable body temperature during activity (homoiothermy) and still be an ectotherm that absorbs heat from the sun (Cowles 1962).

Heterothermy refers instead to a broad range of thermal strategies comprised between ectothermy and endothermy (Boyles et al. 2010). For example, some tuna fish can raise the temperature of their swimming muscle to enhance aerobic performance (local heterothermy), while the body temperature of several mammal species can drop during the night to reduce the difference between internal and external body temperature and hence the metabolic expenditure (temporal heterothermy).

Other terms refer more specifically to the source of heat. A butterfly is likely to expose its wings to solar radiation, in what is called *heliothermy*, while a gecko hidden during the day may press the body against a warm rock, hence displaying *thigmothermy* (Cowles 1962).

It can be easily seen that more than one of these categories may apply to the same individual pending the time of the day, the season or its position in the environment. Unfortunately, without precise measures, it can be quite difficult to assign the proper label. For example, during winter an inactive ectotherm might be considered as a poikilotherm because its body temperature fluctuates with environmental temperature. However, many ectotherms can select refuges based on their temperature, and hence keep a rather constant body temperature even when considered inactive (Huey et al.1989).

In the experiment presented here, I tried to use any label the less possible, apart from ectotherm and ectothermy. The lizard species used here all raise their body temperature by basking in the sun and then shuttling between sun and shade patches. They also take advantage of thigmothermy by pressing against warm rocks, especially during late afternoons in spring and autumn (pers. obs.). Meanwhile, the also contact the ground—often rocks. Hence, even though solar radiation in basking lizards is usually prevalent, I made here no attempt to distinguish the relative contribution of the two factors. Instead, the degree of poikilothermy, that is the degree of fluctuation of body temperature is central here. To understand to what extent lizards' body temperature may change, it is first necessary to discuss how they regulate it.

Box 1: Thermal relationship of an organism with the environment

Life on Earth ultimately depends on the Sun's radiation. Radiant energy from the sun reaches the earth and then flows to the cosmic cold of space. Such a continuous flow of energy from the sun to the cosmic sink is what shapes ecosystems on Earth. Organisms are constantly immersed in this flow of energy, that may occur through radiation, convection, conduction, chemical reaction or mechanical transfer of energy. The relative importance of each of these physical processes depends on the environment and the organism (Gates 1980). For example, aquatic organisms mainly exchange heat with the environment through conduction and convection (Spotila et al. 1992).

Conversely, a ground-dwelling organism like a lizard may be exposed to direct sunlight, as well as reflected light and radiant heat from the surrounding objects. A lizard, depending on the difference between its body temperature and the environment, will also emit radiant energy and exchange energy through convection, conduction and chemical reaction (Fig. 1.4.1). For example, if the body of the lizard is in contact with the ground, it will exchange energy in the form of heat. The flow direction will depend on the temperatures of the two surfaces in contact. If the soil is warmer than the lizard, the heat will flow from the ground to the lizard. Other mechanisms like respiratory cooling and panting may contribute as well to regulate the body temperature of an organism. A lizard can then wander around seeking the spot that provides the best conditions to attain and keep its body temperature close to a preferred value, a behavior that is often referred to as *thermoregulation*.

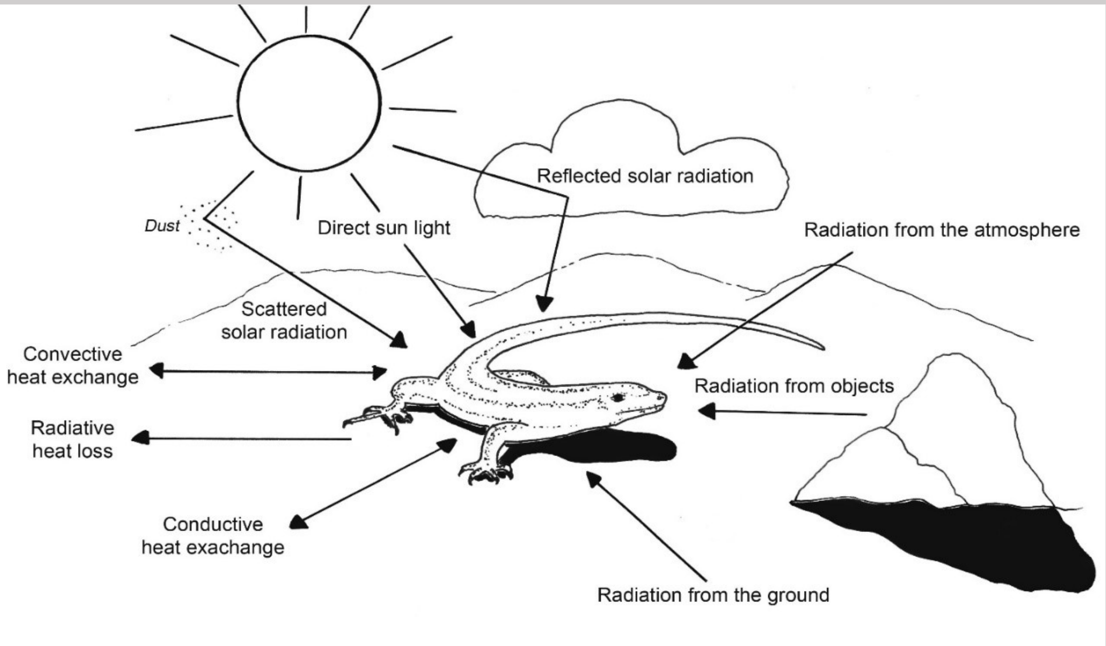

Fig. 1.4.1. Thermal relationships of an organism with the environment. Even though an ectotherm is pictured, the same relationships apply to endotherms. Note that while in some cases energy flows unidirectionally, in others the flow will depend on the temperature difference between the organism and the surroundings. Credit: *Marco Sannolo*.

1.4 The regulation of body temperature in ectotherms

"*Reptiles under the sun - Popularly regarded as the world's most hardened sun-baskers, recent investigations show that most desert reptiles succumb in a matter of minutes to temperatures which man finds only moderately uncomfortable*" (Bogert 1939).

The sentence that opened the seminal work of Bogert was the onset of the research on thermal ecology of reptiles, and still nowadays inspires new investigations. For hundreds of years reptiles have been considered cold-blooded even by researchers, and Linnaeus (1758) grouped them with amphibians (the genus *Lacerta* comprised both *Lacerta palustris*, the common newt, as well as *Lacerta teguixin*, the gold tegu). It was only when researchers started to measure the field body temperature of reptiles that became clear that reptiles *thermoregulate*, that is they can maintain their body temperature at different, often very high, values than air temperature without producing metabolic heat (Cowles 1940; Cowles and Bogert 1944).

During the following years a large amount of data became available for most of the reptile groups, showing that field body temperature varies depending on the species considered, time of activity, season, elevation and ecology among others (Bogert 1949; Brattstrom 1965). Heath (1964) was the first in criticizing the uncritical comparison of body and air temperature by showing that beer cans placed in the field may reach temperatures much higher than the surrounding air, and quite similar to those of reptiles. The understanding that even the temperature of inanimate objects may differ from that of the air eventually led to reframing reptilian thermoregulation in a mechanistic and biophysical context (Porter and Gates 1969; Gates 1980). The concept of operative temperature was ideated in this context of biophysical modeling (Bakken 1980; Bakken et al. 1985) and eventually allowed to establish a protocol to evaluate if and to what extent an ectotherm is thermoregulating (Hertz et al. 1993; Blouin-Demers and Weatherhead 2001). Nowadays, the concepts and mechanisms of reptilian thermoregulation are still widely used, often to assess and predict the effect of climate change and the thermal heterogeneity of a habitat (Sinervo et al. 2010; Sears et al. 2016).

By behaviorally exposing to or retreating from sun's radiation, ectotherms can modulate their body temperature to a much greater extent than endotherms (BOX 1). Such a possibility translates into a vast range of possible body temperatures available to ectotherms, which have been often classified from perfect thermoconformers to perfect thermoregulators depending on the relationship between body and environmental temperature (Huey and Slatkin 1976). Most species may vary the degree to which they regulate their body temperature. For example, during the cold night in temperate climates, many reptiles are usually inactive inside refuges and their body temperature approximate that of the surrounding environment (Brattstrom 1965; Peterson 1987). On the contrary, even fossorial species often considered thermoconformers like *Blanus cinereus* or many gecko species can thermoregulate their body temperature by changing the depth during the day or by hiding below warm rocks (López et al. 1998; Kearney and Predavec 2000).

When active above ground, a reptile is often exposed to solar radiation approaching from different angles, even during cloudy days (BOX 1). Behavioral thermoregulation implies that reptiles can perceive their body temperature and move through the surroundings to adjust it and keep it as close as possible to a preferred value (Hertz et al. 1993). At the cellular level, the temperature is sensed by heat-sensitive transient receptor potential (TRP) channels present in many cell types (Jordt et al. 2003). Such sensors are widespread in all tissues, including skin. Thanks to these receptors, humans can perceive with their skin the heat radiating from an object and leeches use them to select for warmer waters when digesting (Konietzny 1984; Petersen et al. 2011). The input from TRP in vertebrates is conveyed by afferent neurons and are processed in the brain, mainly in the hypothalamus (Morrison and Nakamura 2011). Other organs, like the pineal gland, are involved as well in thermoregulation in some animal groups (Ralph et al. 1979). The processed thermal information is then used to calibrate and direct the process of thermoregulation, either behaviorally (i.e. shuttling) or physiologically (i.e. sweating).

1.5 Water balance and the relation with body temperature in reptiles

Liquid water is, together with temperature, fundamental to sustain life. Indeed, space explorations searching for exoplanets with environmental conditions suitable for life look out for mild temperatures associated with liquid water (McKay 2014). The physical and chemical properties of water are well studied, as well as the relationship between such properties and temperature (Clarke 2017). The importance of liquid water is so fundamental to support life that extensive research has been carried on this subject. For example, we know that metabolic active cells contain ~70% of water (Ling 2004) and that the total water content of animals ranges from 54% in insects to over 96% in gelatinous zooplankton (Studier and Sevick 1991; Kiørboe 2013). Such a considerable variation in water content reflects underlying phylogenetic structures, as well as anatomical and physiological adaptation, with some animal groups more linked to water or moist environments than others (Lillywhite 2006). Hence, in those animal groups more resistance to water loss, like reptiles, research has been mainly focused on the physiological and anatomical adaptations that enable them to enhance water loss resistance, like the lipid content in the skin (Roberts and Lillywhite 1980) or an efficient excretion system (Minnich 1970). Not surprisingly reptiles have been considered extremely resistant to water loss because their skin is little permeable to water and their excretion is not soluble (Bentley and Schmidt-Nielsen 1966). In fact, the integument of several snake and lizard species is less resistant to dehydration than human skin (Lillywhite 2006).

Osmoregulation in reptiles is well studied, especially regarding the physiological process and anatomical adaptations (Mautz 1982). The main avenues of water intake in reptiles include drinking and feeding, while absorption across the skin is almost irrelevant, even though some desert-dwelling species like the agamid lizard *Moloch horridus* use integumentary structures to convey water to the mouth (Bentley and Blumer 1952; Krakauer et al. 1968). Conversely, reptiles lose water in feces and urine, and by evaporation from the skin and the respiratory tract (Shoemaker and Nagy 1977). Despite the common belief that the low permeability of reptilian skin relies on the presence of scales and a thickened keratinized epidermis, several studies have shown that epidermal lipids are more likely playing a fundamental role in waterproofing reptile skin (Roberts and Lillywhite 1980; Hadley 1989). The relative contribution of each of these avenues depends on many variables—and often their interactions—like body temperature, the species considered and the context. For example, rates of evaporative water loss are usually higher in lizards from populations of the same species that live in more humid environments (Hillman et al. 1979). Similarly, digestive efficiency not only increases exponentially with body temperature (Adolph and Porter 1993), but it also depends on the diet, with insectivorous lizards being more efficient than herbivorous ones (Qu et al. 2011).

For reptiles, much less information is available on the relationship between water balance, thermoregulation and activity. Indeed, most of the studies and reviews on this subject focus on desert reptiles (Brattstrom 1965), because they represent an adaptation to extreme environmental conditions (Cloudsley-Thompson 1991). The traditional belief that reptiles are virtually unaffected by water loss led to understudy such ecological relationships, especially in temperate and tropical species. Similarly, most of the investigation on the potential effects of climate change on reptiles focuses on temperature (Huey et al. 2009; Huey et al. 2010; Sinervo et al. 2010). In recent years, however, interest has surged to study reptilian thermoregulation and water balance within the same conceptual framework (Guillon et al. 2013). Such an interest is strengthened by evidence that suggests that in the next decades, reptiles will not only face changes in mean environmental temperatures but also in terms of extreme temperature fluctuations and water availability and rain regimes (Schär et al. 2004; Dai 2013; Urban 2015).

Even though comparably fewer studies investigated the potential effects of dehydration on physiology and behavior, the available literature indicates that body temperatures and activity are generally negatively affected in dehydrated lizards. For example, dehydrated lizards select lower body temperatures in thermal gradients (Crowley 1987), and their endurance is reduced under dehydration (Wilson and Havel 1989). Similarly, individuals exposed prolonged drought are more likely to refrain from activity and spend more time hiding than individuals with access to water (Ryan et al. 2016). The issue of how dehydration affects thermal preferences is further explored in Article V. At the physiological level, suffering dehydration in early life staging may lead to lower growth rate and lower activity level (Lorenzon et al. 1999). Populations as well may vary in their sensitivity to dehydration. For example, lizard populations with limited access to free-standing water are more resistant to water loss than those with full access to water (Dupoué et al. 2017). Still, it is largely unknown to what extent water loss resistance may differ in sympatric species or its variability along environmental gradients, like altitudinal slopes. Some of these aspects are investigated more in detail in Articles III and Articles VI.

1.6 Thermal ecology studies are geographically —hence taxonomically— biased

A century of Hollywood movies influenced worldwide people behavior, perception and evaluation of new movies (Glantz 2001; Anderson and Bushman 2002). Something similar can be said about U.S.A. fast-food (Popkin and Gordon-Larsen 2004; Schlosser 2012). And thermal ecology studies. Even though it was known since the ancient Greeks time that temperature profoundly influences life and health (Kleisiaris et al. 2014), only at the beginning of the past century researchers started to study rigorously the relationship between body temperature and animal physiology (Barcroft and King 1909; Hall and Root 1930). The first studies on reptile ecophysiology, body temperature and activity were carried on desert species (Buxton 1924), usually in the U.S.A. (Cowles and Bogert 1944). The trend continued for several years, and the first review available on reptile body temperatures and heat tolerance comprised mainly North and Central American species, many of which inhabiting desert regions (Brattstrom 1965). Far from being a historical triviality, still nowadays the first evidence that climate change may lead to local extinction in lizard populations come from desert areas (Sivervo et al. 2010). Similarly, comparatively few studies investigated thermal ecology of tropical species, which are by many considered more exposed to climate change and risk of extinction (Huey et al. 2009).

One of the results of such an asymmetry —geographical and taxonomical— in thermal ecology studies is, for instance, that no research was carried on the thermal ecology of lacertid species living temperate regions like Europe before the ’80 of the previous century (Rismiller et al. 1985). Hence, the potential adverse effects of the current climate change on survival, range shift and adaptation are challenging to predict or measure because we lack the necessary information on the ecophysiology of most ectotherm species inhabiting temperate and tropical regions. For example, mechanistic models incorporate physiological data to increase the robustness of their prediction (Leibold 1995; Kearney and Porter 2009). However, a significant impediment to the development of mechanistic models is the availability of accurate and comprehensive data on behavioral and physiological traits. Such data are economically expensive to get and require extensive field and laboratory work. Not surprisingly, in recent years cheaper correlative models have become very popular (Elith and Leathwick 2009), even though their poor predictive performance in space and time has been often criticized (Pearson and Dawson 2003) and the theoretical basis to develop robust mechanistic models were laid down several decades ago (Porter and Gates 1969; Bakken 1980; Gates 1980; Bakken et al. 1985).

1.7 Thermal and water ecology studies on Lacertid lizards

The family Lacertidae is a monophyletic group that nowadays comprises 335 species grouped within 44 genera, a number that is likely to increase further in the next future (Pyron et al. 2013; Uetz, 2019). Lacertids are small to large body-sized lizards, ranging from 40 to 260 mm adult SVL and the family is widely distributed in the Old Word, with representative living in most Africa, Europe and Asia southward into the northern East Indies (Vitt and Caldwell 2014). Lacertids have been intensively studied under most biological aspects, spanning from systematics (Arnold 1973; Harris 1999: Arnold et al. 2007; Pyron et al. 2013) to morphology (Vanhooydonck and Van Damme 1999), physiology (Díaz et al. 1996; Vanhooydonck et al. 2001), ecology (Arnold 1987; Perry et al. 1990) and distribution (Sindaco and Jeremčenko 2008; Sillero et al. 2014).

Nonetheless, the first studies on the thermal ecology of lacertid lizards came late if compared with the already available information on phrynosomatid and iguanid lizards, and still focused on desert-dwelling species (Duvdevani and Borut 1974). The first revision of the thermal ecology data available for lacertid lizards came a few years later (Avery 1982). Even though a good wealth of knowledge is available on the thermal preferences of several lacertid species (Bauwens et al. 1996; Castilla et al. 1999; Meiri et al. 2013). Hence, it is not surprising that still nowadays several lacertid species lack a comprehensive compendium on their thermal physiology and ecology, either because some species have been recently divided into new ones (Harris et al. 2007), or just because a few common and attractive species have monopolized most studies on lacertid thermal ecology. Thus, one of the reasons to study this group of lizards is the lack of primary data on their thermal ecology, that severely limits our understanding of their biology as well as impedes us to produce reliable predictions on the potential negative consequences of habitat loss and climate change. Furthermore, many of the under-studied species live in remote or even highly politically unstable areas, making such studies more challenging. For example, the genus *Darevskia*, comprising nowadays 32 species (Uetz 2019) lacked any data on thermal ecology until very recently (Ćorović and Crnobrnja-Isailović 2018). Another, specular reason, to focus on this lizard group is the availability of many detailed data on a few well-studied species that have risen to the status of a model organism to ask specific questions on thermal ecology processes and patterns. In this aspect, some of the best-studied species belong to the genera *Podarcis*, *Lacerta* (including *Timon*), *Psammodromus* and *Zootoca*. The present work followed this latter approach, by taking advantage of the previously available knowledge on some species belonging to these groups to investigate specific biological question on their thermal ecology.

Water balance, instead, is virtually uncharted territory for most lizard groups, including Lacertidae. Some of the reasons have been already discussed, like the traditional belief that reptiles are unaffected by water loss and the late onset of ecophysiological studies on lacertid lizards. In recent years, however, interest has surged in studying water balance and thermoregulation within the same context. Before the present work, a few studies compared differences in water loss rates in sympatric lacertid species (García-Muñoz and Carretero 2013; Osojnik et al. 2013; Carneiro et al. 2015), its variability at the population level (Dupoué et al. 2017), and the influence of water during development (Lorenzon et al. 1999).

1.8 *Podarcis, Timon* and *Psammodromus* thermal and water ecology

The genus *Podarcis* currently comprises 23 described species widely distributed in Europe, NW Anatolia and NW Africa (Sindaco and Jeremčenko 2008; Uetz, 2019). Even though the systematics of this genus has been investigated extensively, it is still not completely resolved (Harris et al. 2005). Some group of species, like the *P. hispanicus*-complex are particularly difficult to disentangle (Pinho et al. 2008; Kaliontzopoulou et al. 2011a), and species recognition using morphology may be arduous, if not impossible (Kaliontzopoulou et al. 2011b). *Podarcis* is likely one of the best studied genera within Lacertidae family. Several studies investigated the thermal preferences and thermal limits of many representative of this genus (Van Damme et al. 1990; Castilla et al. 1999; Gvoždík and Castilla 2001; Veríssimo et al. 2009).

Despite being relatively well studied, the unresolved systematics of the genus *Podarcis* led in recent year to raise several forms to the species level. The most emblematic case is represented by *P. hispanicus*-complex, in which many forms have been raised to the specific status in recent years (Renoult et al. 2010; Kaliontzopoulou et al. 2011a; Geniez et al. 2014). For several of these forms there is scarce data on thermal biology. This is not necessarily due to the lack of investigation; rather, oldest research often pulled together populations or individuals from areas in which nowadays more than one species inhabits. Several *Podarcis* species inhabiting the Iberian Peninsula show interesting pattern of allopatric divergence, with a few cases of species living in sympatry (Kaliontzopoulou et al. 2011a). For most of *Podarcis* species, scarce data is available on the water ecology. However, a few species have been investigated in this aspect (García-Muñoz and Carretero 2013; Osojnik et al. 2013; Carneiro et al. 2015), generally showing that *Podarcis* species are quite sensible to water loss. The comparison of the thermal preferences and resistance to water loss in closely related sympatric species is investigated further in Article III and IV. Instead, to what extent water loss may influence thermal preferences is examined in Article V.

The genus *Timon* was once included inside the genus *Lacerta* (Arnold et al. 2007) and nowadays comprises six species, distributed non-continuously in the Western Mediterranean basin and Middle East (Sindaco and Jeremčenko 2008). *Timon* species are among the biggest lacertid lizards, reaching up to 240 mm adult SVL (Mateo and Castroviejo, 1991). The best studied species of the genus is *Timon lepidus*, for which many studies are available on its ecology (Hódar et al. 1996, Mateo 2014). Similarly, the thermal ecology of *Timon lepidus* is relatively studied, while for the other species of the genus data on thermal preference is scarce. *T. lepidus* is a territorial and thermophilic species, which is active over a broad range of body temperatures (Busack and Visnaw 1989). Instead, to the best of my knowledge, very little information is published on the water balance or on the resistance to water loss in this genus (Ferreira et al. 2016). Here, Article I will focus on aspects *T. lepidus* thermal preferences, will in Article II I will explore the consequences that hiding from a predator may have on the body temperature of this species.

The genus *Psammodromus* comprises six species inhabiting the Western Mediterranean (Sindaco and Jeremčenko 2008; Uetz, 2019). The biology of *Psammodromus algirus* has been intensively studied. It is among the most thermophilic lacertid species along with *Acanthodactylus*, selecting approximately 35°C in laboratory thermal gradients (Díaz 1997), while the body temperature of active individuals changes little with altitude and is approximately 33°C (Zamora-Camacho et al. 2013).

As far as I am aware of, almost no data is available on the water ecology of *Psammodromus algirus*, likely because it is considered a species resistant do desiccation and, hence, most of the research focused on its thermal biology (Ferreira et al. 2016). Thus, study the relationship between thermal ecology and water balance in this species would not only be novel, but will also provide essential information to understand the biology of this species. Article VI assess the water loss rate for this species and explores the potential variability of this trait across populations and along an altitudinal gradient.

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Chapter 2: Objectives and Thesis structure

“Nobody is driven by abstractions like 'seeking truth.”

— Michael Crichton, Jurassic Park (1993)

2.1 Detailed objectives

In the present thesis, I investigated the thermal and water ecology of lacertid lizards using a variety of species, approaches, methods and goals. The main aims of the current work were to assess the efficiency and promote the use of cutting-edge IR technology in ectotherms' thermal biology, to study interspecific differences in thermal ecology, water balance and performance, and to investigate the role of dehydration in modulating lizards' thermoregulation, both in the lab and in free-ranging lizards. All species used were lacertid lizards from various *genera*, even though I believe that the same questions may apply to many other ectotherm species.

As explored in the method section, Infrared Thermal Imaging is one of the methods I used most extensively to record lizards' body temperature. While such a method is already occasionally used in herpetology, at the time I started this thesis, no rigorous calibration was available to compare it with more traditional protocols like cloacal measurements. Hence, one of my first objectives was to support the use of IRT as a valid, non-invasive alternative to other methods used to record lizards' body temperature. Once IRT was confirmed to be a robust method, I wanted to test it in the field. I did not skip a laboratory test. I already conducted in the past an experiment in which I demonstrated, using IRT, that small lizards have significant differences in temperature depending on the body part. Hence, it was time to demonstrate that IRT can be used in the field to investigate interesting biological questions on ectotherms. Therefore, the aim of the first two articles of this thesis was not only to demonstrate that IR technology provides comparable results with other methods, but that it can also expand the range of biological questions that can be answered using such a technology.

The second aim of this thesis was to investigate interspecific differences in ectotherms thermal ecology. I was particularly interested in exploring if closely related species living in syntopy may differ and to what extent. Hence, the following two articles explore and discuss differences in preferred body temperature, resistance to water loss and performance in two small lizard species. As already explored in the introduction, studies that integrate water balance and thermoregulation are known becoming more common, and during the thesis, I realized that dehydration is likely one of the most critical factors affecting thermoregulation in lizards. Thus, in half of the articles presented here water share a starring role along with temperature.

Water is so essential for small lizards that the last two articles investigate if and to what extent water balance and thermoregulation may interact. First, I conducted a laboratory experiment to test if dehydration negatively affects thermoregulation and to measure to what extent thermoregulation is impaired in dehydrated lizards. Then, I investigated in the field if thermoregulation and water balance vary among populations, since different populations may experience various thermal and water availability regimes, especially along defined environmental gradients, like an altitudinal slope.

2.2 Thesis structure

This thesis is divided into eight chapters. After this section, in Chapter 3 I will extensively explain the main methods used throughout the manuscripts that compose this work. The following three chapters are each divided into two articles, either already published or submitted for publication. Chapter 4 will explore IR as a method to obtain lizards' body temperature and then use this method in the field. Chapter 5 is focused on the interspecific comparison of thermal ecology, resistance to dehydration and performance using two sister species. Finally, Chapter 6 explores more in detail the relationship between thermal ecology and water balance. Chapter 7 will provide an extensive discussion on the results obtained developing this thesis, while Chapter 8 will provide the supplementary material for all papers.

2.3 Chapter 4: Thermal Imaging in lizard studies and field application

Article I: Assessing the reliability of thermography to infer internal body temperatures of lizards

Even though infrared imaging has been already used in few cases to gain insight on lizard body temperature, it has never been calibrated against other traditional methods of temperature recording, like cloacal measurements. Hence, the objective of this work was to compare IRT data obtained from various body parts against cloacal measurements obtained with a probe. Furthermore, since body size may affect the readings, I used three species of various size to test for such an effect and generalize the conclusions. Finally, I checked for possible differences between the heating and cooling phase. The results might not only support the use of IRT for future studies but will also allow a direct conversion between cloacal and IRT measures.

Article II: Waitin' on a sunny day: factors affecting lizard body temperature while hiding from predators

Lizards have been used extensively to study hiding strategies. However, the thermal cost of hiding into a thermally unsuitable refuge has never been investigated in depth. Here I took advantage of infrared imaging to study for the first time in the field the thermal cost of hiding in a lacertid lizard. I took thermal pictures of free ranging lizards before simulating a predation attempt and again after the lizard emerged from the refuge. With this protocol I were able to calculate for the first time the thermal cost of hiding in wild lizards.

2.4 Chapter 5: Thermal ecology, water balance and performance curves of sympatric sister taxa species of lacertid lizards

Article III: Physiological differences in preferred temperatures and evaporative water loss rates in two sympatric lacertid species

This chapter is the first of the two that focus on comparative physiology, using small sister species of small body-sized lizard as case study. In this work I was interested in comparing the preferred body temperature and the resistance to water loss rate of two species closely-related in the phylogeny and often found in syntopy, *Podarcis bocagei* and *Podarcis guadarramae lusitanicus*. I assessed preferred body temperature using standard thermal gradients, and measured resistance to water loss rates measuring the change in body mass through time. The results provide useful insight on how closely related species can live together while reducing their niche overlap.

Article IV: Is suboptimal optimal? A test of Jensen's inequality using wall lizards

Having assessed in the previous work the physiological differences existing between *P. bocagei* and *P. g. lusitanicus*, I tested here the hypothesis that ectotherm species may select suboptimal body temperatures even though their performance are better at higher body temperatures. Lizards of both species were made run on a standard track at various temperatures, while being recorded with a high-speed camera. Their maximum speed was then extracted and plotted against body temperature. The performance was then compared with data on thermal preference obtained in thermal gradients. The results may shed light on lizard thermoregulation and thermal physiology.

2.5 Chapter 6: The effects of dehydration on thermal preference and intraspecific variability of dehydration resistance

Article V: Dehydration constrains thermoregulation and space use in lizards

This chapter focuses on the interaction between thermoregulation and water balance at the individual and specific level. In this first work I used four species of *Podarcis* lizards to test if dehydration may negatively affect thermoregulation, assuming that dehydrated lizards would be forced to thermoregulate less precisely, reduce their body temperature below the preferred values and changing how they use the surrounding space. Lizards were given the chance to thermoregulate in standard thermal gradients with or without a source of water and a refuge, while their body temperature was regularly recorded. The results are of particular relevance to understand how two fundamental physiological aspects, namely thermoregulation and water balance, interact and to understand to what extent dehydrated lizards change their behavior in the field.

Article VI: Variation in field body temperature and resistance to dehydration along an environmental gradient in a diurnal ectotherm

In this last paper I tried to move to the field the investigation on the interaction between water balance and thermal ecology. While data on lizard's thermoregulation are abundant, much less is known on water balance, and very scarce information is available on intraspecific variability. Here I used as model the lizard *Psammodromus algirus*, a species that prefers high body temperature and is considered very resistant to water loss. I monitored three populations, recording individual body temperature, sex, location and altitude and then testing in the lab for water loss rates. The results are the first available for water loss resistance in this species, and the comparison of the three populations will allow discussion on factors modulating water balance at the local scale.

Chapter 3: General Methods

"It is common sense to take a method and try it. If it fails, admit it frankly and try another. But above all, try something."

— Franklin D. Roosevelt (1932)

3.1 Methods in thermal ecology

The chapters that follow are published in or submitted to international peer-reviewed journals. Editorial guidelines can be painfully strict regarding the length of a manuscript, and authors are frequently forced to shrink as much as possible each section. As a result, often the material and methods section of a paper ends up being an unintelligible block of jargons that likely will discourage any researcher to try to replicate that experiment. Fortunately, a PhD thesis does not have a word limit (at this point the reader is already suffering the negative consequences of such limitlessness). This section is thus intended to provide a reference base, historical as well as methodological, and a broad discussion of the most used methods in the following chapters.

3.2 Infrared Thermal Imaging

In the present work, Infrared Thermal Imaging (IRT), also called *Thermography* for short, will be used extensively. Indeed, of the six Articles that compose the research body of this thesis, IRT is used in four of them. In the other two Articles, lizards' body temperature was measured with a cloacal probe for practical reasons or, as in Article III, to merge and compare different data sets. Here I will briefly describe infrared radiation, how it is measured, its applications and why I decided to rely so heavily on such a technology.

Infrared radiation (IR) is electromagnetic radiation (EM) characterized by those wavelengths comprised within the visible and the microwave spectrum. More specifically, IR wavelengths range between 0.78 µm and 1 mm, even though only a fraction of the IR wavelengths is used in thermal imaging (Fig. 3.1). Usually, three spectral ranges are used in IRT: the long-wave (LW) region from around 8 to 14 µm, the mid-wave (MW) region from around 3 to 5 µm, and the short-wave (SW) region from 0.9 to 1.7 µm. On which IR region one should focus, and hence which camera to select, depends on the research aims. For example, for industrial applications where the temperature of extremely hot materials has to be measured, SW cameras give the best accuracy. At temperatures of biological interest, generally comprised between 0 and 40°C, MW cameras provide the best performance (Möllmann and Vollmer, 2017). However, MW cameras remains nowadays very expensive, at least for the average research budget. Moreover, commercial and portable cameras are invariably LW cameras. Hence, a trade-off between maximum performance and affordability and portability is usually needed. In the present thesis, a FLIR T335 camera has been used in all studies. The camera spectral range is comprised between 7.5 and 13 µm, thus covering most of LW radiation.

Any object with a temperature T > 0 K emits some EM radiation. For objects at a temperature of biological interest, such radiation is in the IR spectrum. Hence, Thermography exploits such physical properties by detecting an object's thermal radiation and translating it into temperature values that can be used. Because most solids are opaque to IR radiation, Thermography detects only superficial temperatures, that is the thermal radiation emitted at the outer layer of the object in contact with the transmitting medium (usually the atmosphere). Even though such point might seem obvious, external temperature of an object, like a lizard, may not be the same of its core. Such a hypothesis and its practical consequences when measuring lizards' body temperature laid the ground for the first article of this thesis. When measuring the temperature of an object, like the lizards used in this thesis, many variables can affect the reading and hence must be accounted for. Some parameters can be adjusted after a thermal picture is taken by using appropriate software. For example, here *FlirTools* has been used to consider the emissivity of lizards' skin, as well as the distance between the operator with the subject, and the temperature and humidity of the ambient.

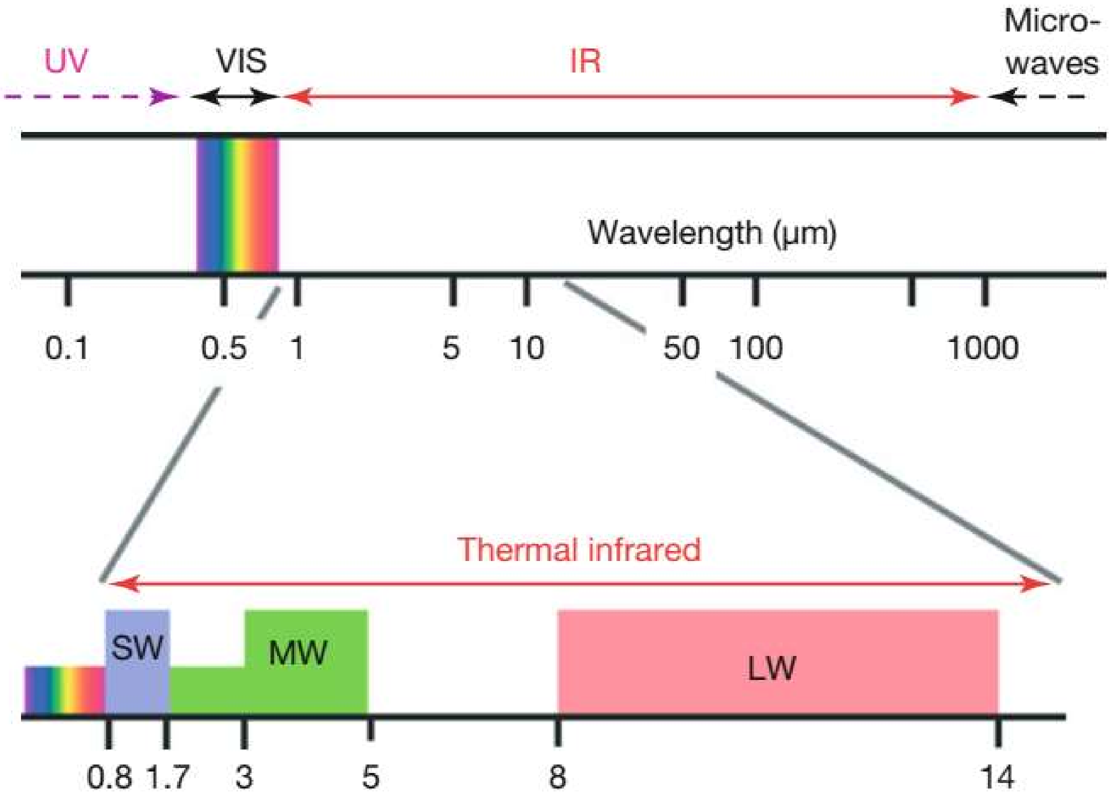

Fig. 3.2.1. Spectral regions of the light from the UV to microwaves. IR radiation is comprised between 0.78 μm and 1 mm. Within this range, thermography systems are specially developed for short-wave (SW), mid-wave (MW) or long-wave (LW). Adapted from Möllmann and Vollmer (2017).

Several other factors may significantly affect the analysis of thermal pictures and cannot be readily accounted for using a software, like the angle of observation, the use of filters, thermal reflections, wind speed and solar load, among others. Thus, I tried to standardize as much as possible all thermal pictures taken. For example, shooting distance within each study was kept constant, as well as the angle at which the photo was taken. Similarly, in the laboratory, ambient temperature and humidity were kept constant throughout the study, while in the field such strict control on ambient parameters was just not possible.

Thermography is a rapidly expanding field, primarily due to the development in the last decade of both more affordable and portable thermal cameras. In non-biological fields, it is for example extensively used for monitoring and diagnosing the status of building and infrastructures (Kylili et al. 2014), in microsystem engineering (Möllmann et al. 2004), as well as in industry for predictive maintenance, condition monitoring and quality control (Schultz 2004; Bagavathiappan et al. 2013) and possibly even to track the thermal signature left behind by nuclear submarines while sailing close to the sea surface (Daniel 1986).

In Biology, Thermography is used in the clinical context both in humans (Diakides et al. 2013) as well as in animal medicine (Rekant et al. 2016). Recent applications include criminology as well (Moliné et al. 2018). In contexts more relevant to this thesis, Thermography is being used for many years. To the best of my knowledge, Jones and Avery (1989) were the firsts in trying to measure lizard body temperature using Thermography under laboratory condition. Nowadays, Thermography has been already used for example to show for instance that the toucan bill may act as a thermal radiator (Tattersall et al. 2009) and that the facial pits of many viperid species are kept at a lower temperature than the surroundings to improve sensor accuracy (Tattersall and Cadena 2010). In recent years, I was able to contribute a little on the subject by showing that not only desert lizards are able to create a difference in body temperature between the head and the trunk (Heath 1964), but also lacertid lizards show similar patterns (Sannolo et al. 2014).

3.3 Preferred body temperature

To the best of my knowledge, Cowles and Bogert have been among the first researches bringing thermocouples in the field to measure reptiles' body temperature during the '30 and '40 of the past century (Bogert 1939; Cowles and Bogert 1944). These two researchers worked and published together in many occasions, and they referred to “preferred temperature” as the average body temperature recorded periodically of an animal kept in a cage and exposed to field condition. The definition of what is preferred body temperature and how to measure it evolved during the last 70 years (Camacho and Rusch 2017) and in the present work I defined preferred temperature as the *body temperature that an organism selects in an environment that lacks physical and ecological constraints on thermoregulation* (Porter and Gates 1969; Huey and Slatkin 1976)

Preferred body temperature is usually measured in an artificial context in which any constraints can be removed, and the thermal landscape modified at will. The first step is usually to build an area, that can be linear (Licht et al. 1966), circular (Bowker 1984) or patchy (Pulgar et al. 1999). Here, the same linear gradients have been used in all experiments (Fig. 3.3.1). To create a uniform gradient of temperature, a light bulb of 150 W was suspended over one end, while the air conditioning system was set no ~18-20°C. With this setting, I was able to create a thermal gradient of operative temperature ranging between 20 and 50°C across the arena.

Once a lizard is acclimatized to the experimental environment (usually after one day), its body temperature has to be measured periodically, and several methods exist to get such data. Typically, and most commonly, cloacal temperatures are measured with a thermometer fitted with a thermocouple. Depending on the species, the thermocouple is inserted few millimeters or even a centimeter inside the cloaca, and the reading is taken as soon as the temperature stabilizes. I used here such a method in Article I (using K-type thermocouple), while in all other cases, a thermal camera (FLIR T335) was used to record body temperatures. The use of different measurement devices was dictated by the need of comparison of some data collected before the present work started. Body measurements can be taken as often as every few minutes (Licht et al. 1966). Here I preferred to measure body temperatures every hour to eliminate or at least reduce as much as possible the temporal autocorrelation in the data and to allow comparison with previous studies.

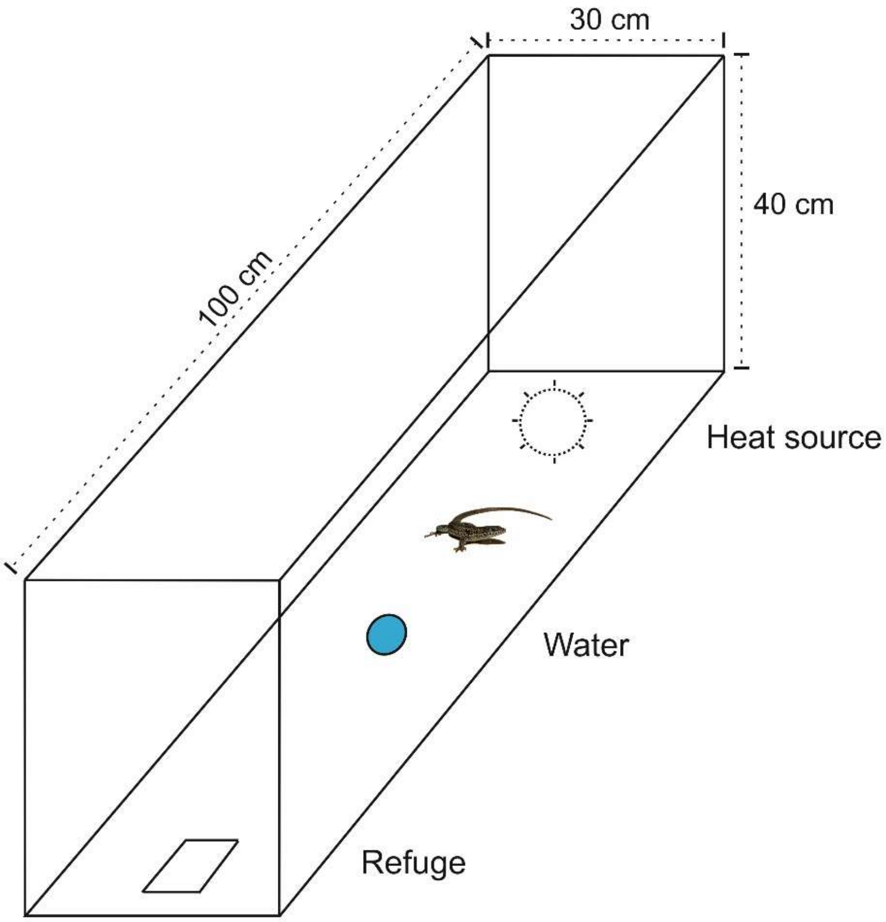

Fig. 3.3.1. Example a thermal gradient used in this thesis. The gradient is built in PVC, heat is provided buy a 150 W lamp positioned above one end. Temperature range from ~50 to 20 °C is obtained by setting appropriately the air conditioning system in the room. Lizards had also access to water (but see Article V) and a refuge. Body temperatures were recorded every hour (Author: *Marco Sannolo*).

Once data on the preferred temperature are gathered, the next step is to describe the preferred temperatures statistically, and eventually compare them across populations and species. “Set-point ranges” can be obtained from the mean or median body temperatures and the most used values are the central 50 or 80% of the data (Hertz et al. 1993; Bauwens et al. 1996). Measures of central tendency, like the mean and the median, are used widely in the literature (Sokal and Rohlf 1995), especially for comparisons among groups. However, it should be acknowledged that not only field body temperatures are usually non-normally distributed and left-skewed (Huey and Pianka 2018), but often also the laboratory estimates of preferred temperatures show non-normality (Sinervo and Adolph 1989). The consequence is that mean values tend to be underestimated in a left-skewed dataset (see Article V).

Since most ectotherms body temperatures distributions do not follow a normal curve, even bigger challenges must be faced when one is interested in comparing the whole distribution of temperature data. One way to address such a statistical obstacle is to try to fit several models to the same dataset and then compare the fit of the models (Burnham and Anderson 2002; Angilletta 2006). Unfortunately, not only thousands of models exist among which to choose, but the best statistical model could overfit the data and perform poorly when applied to a new dataset (Hawkins 2004). Finally, in most cases several repeated measures of body temperature are taken from the same individual during a testing day, raising the problem of appropriately accounting for individual variation. In all the articles presented here, I partitioned the variance in the data by adding the individual identity as a random effect in the models.

Such a variety of experimental procedures, measurements tools and statistical methods give researchers on the one hand, a vast flexibility to collect thermal data and describe the thermal preferences of virtually any species. On the other hand, however, comparisons among studies are more challenging and often unpractical and can be carried out only when the methods used are the same.

3.4 Total Evaporative Water Loss

Reptiles lose water from various avenues, namely through the integument, through respiration and by expelling urine and fecal residues (Hill et al. 2012). Despite the long-standing belief that reptilian skin is practically impermeable to water loss, several studies demonstrated that cutaneous water loss in reptiles might account for as much as ~90 % of the total water loss (Bentley and Schmidt-Nielsen 1966). Indeed, the skin resistance to water loss of many species of lizards living in mesic and xeric environments, like some agamids, is lower than that of human skin (Lillywhite 2006).

The articles presented in this work focus on the sum of respiratory and cutaneous water loss often referred to as the Total Evaporative Water Loss or, for brevity, EWL (Mautz 1982). For practical reasons, ocular water loss and excretory expenditures are not considered here. However, it should be stressed that the experimental protocol used allowed to retrieve dry solid residues, if present, and account for their weight.

To estimate EWL several methods can be used, with a variety of approaches depending on the species, the biological question and the source of evaporation considered (Mautz 1982). In the most straightforward experimental design, that is the one used here, animals are held in the laboratory and exposed to similar environmental conditions. Then EWL is estimated as the loss of body mass over some amount of time. In the present work, lizards were exposed to either one or several constant environmental temperatures inside a thermal chamber, and their body mass was either checked at regular intervals (Article III), or as the difference between the initial and final body mass (Article VI). The obtained data representing mass loss was then divided by the initial mass of each lizard to account for the interspecific and intraspecific difference in body mass. To estimate EWL other more complex designs can be used as well. For example, the cutaneous and respiratory component of EWL can be estimated separately for the same individual within a trial by suspending the animal at the neck in a chamber divided by a rubber membrane. The head and body may be then exposed to different streams of flowing air and sealed in the chamber with a desiccant (McDonald 1976). Such an experimental setting has been used whenever one was interested in comparing or differentiate between cutaneous and respiratory water loss. In recent years, the development of tools to measure the dehydration status of human skin have been tested in reptiles to get measures of instantaneous rates of EWL (Berg et al. 2002; Guillon et al. 2013). The results are promising and open new ways to explore ecophysiological relevant questions. Unfortunately, such a technology is still relatively new and highly expensive.

Many physiological, morphological and behavioral factors may influence EWL estimates. For example, feeding status and the phase of shedding cycle are known to affect EWL rates (Cohen, 1975; Zucker, 1980; Kabayashi et al. 1983). In all the Articles presented here, the potential influence of feeding status and acclimation to humidity condition was standardized by making measurements from fasting lizards that have been exposed to the same humidity throughout the study. Similarly, the potential effect of shedding cycle was controlled by restricting measurements to non-shedding animals. Finally, an animal activity may as well affect EWL, as active lizards may lose water at rates three to four times higher than inactive ones (Mautz 1980). Here lizard activity was not checked directly, because animals were confined inside individual boxes within the thermal chamber kept in darkness. However, we restricted the comparisons to individuals of the same sex, similar body size and the same period of the year. Furthermore, we made sure that all experimental animals were exposed to identical conditions.

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Chapter 4: Infrared Thermal Imaging in lizard studies and a field application

“Anyone owning an IR camera will be able to produce nice and colorful images, but most will never be able to fully exploit the potentials of such a camera – and most will never be able to correctly use it.”

— Vollmer and Möllmann (2018)

4.1 Article I: Assessing the reliability of thermography to infer internal body temperatures of lizards

Journal of Thermal Biology 62 (2016) 90–96

Frederico M. Barroso, Miguel A. Carretero, Francisco Silva, Marco Sannolo

Abstract

For many years lizard thermal ecology studies have relied on the use of contact thermometry to obtain internal body temperature (T_b) of the animals. However, with progressing technology, an interest grew in using new, less invasive methods, such as InfraRed (IR) pyrometry and thermography, to infer T_b of reptiles. Nonetheless few studies have tested the reliability of these new tools. The present study tested the use of IR cameras as a non-invasive tool to infer T_b of lizards, using three differently body-sized lacertid species (*Podarcis virescens*, *Lacerta schreiberi* and *Timon lepidus*). Given the occurrence of regional heterothermy, we pairwise compared thermography readings of six body parts (snout, eye, head, dorsal, hind limb, tail base) to cloacal temperature (measured by a thermometer-associated thermocouple probe) commonly employed to measure T_b in field and lab studies. The results showed moderate to strong correlations (R^2=0.84–0.99) between all body parts and cloacal temperature. However, despite the readings on the tail base showed the strongest correlation in all three species, it was the eye where the absolute values and pattern of temperature change most consistently followed the cloacal measurements. Hence, we concluded that the eye would be the body location whose IR camera readings more closely approximate that of the animal's internal environment. Alternatively, other body parts can be used, provided that a careful calibration is carried out. We provide guidelines for future research using thermography to infer T_b of lizards.

Keywords: Thermography, Thermal imaging, Infrared camera, Lizard body temperature, Regional heterothermy

1. Introduction

Body temperature is a fundamental aspect in the ecology and physiology of ectotherms due to its effects on individual growth, survival, reproduction (Huey and Stevenson 1979; Angilletta et al. 2002; Savage et al. 2004), as well as on species density and diversity (Angilletta et al. 2004; Brown et al. 2004; Wiens et al. 2006). Reptiles represent a particularly well studied group in which most species utilise external heat sources and behavioural and physiological adaptations to thermoregulate (Seebacher and Franklin 2005; Tattersall and Cadena 2010). By doing this, many reptiles maintain their body temperatures within a preferred range, often referred to as "set-point range" (Gans and Pough 1982; Hertz et al. 1993) which optimises a variety of metabolic functions (e.g. digestion, locomotion, growth, incubation – Huey and Stevenson 1979; Van Damme et al. 1991). The temperatures and precision to which reptiles thermoregulate is dependent on many factors such as species, sex, age, season, reproductive, nutritional and health state (Gans and Pough 1982; Beal et al. 2014; Gunderson and Leal 2015). Yet, most species can be thought of being somewhere amid a continuum between the perfect thermoregulator and the perfect thermoconformer (Huey and Slatkin 1976), but the position in this scale is neither static nor absolute, but rather a dynamic range affected by many ecophysiological traits (Hertz et al. 1993; Angilletta 2010).

Among reptiles, lizards have commonly been used as model organisms in thermal ecology and ecophysiology studies (Castilla et al. 1999). For example, lizards have been widely used to study thermoregulation patterns (Gunderson and Leal 2015) and adaptation of thermal niche (Aguado and Braña 2014; Ma et al. 2014), effects of current climate change on geographic distribution (Bestion et al. 2015; Woods et al. 2015), and preferred body temperature and thermal heterogeneity (Allen and Powell 2014; Goller et al. 2014).

The previous studies resulted in well-established protocols for collecting data, such as the use of cloacal probes to measure body temperatures. Such wide adoption of this procedure is the results of the ease of applying this tool in both field and lab conditions. However, the increasing perception of the complexity behind reptile thermal ecology led to a growing interest in developing new tools and techniques to investigate this aspect of reptilian ecology.

For example, some studies have demonstrated the utility of the use of infrared (IR) technology as an alternative to contact thermometers to measure body temperature. The use of IR technology, such as IR thermometers (pyrometry) and IR thermal imaging cameras (thermography), allows to collect temperature data without the need to capture the animal, and often with great speed, short lag and in high-resolution (Hare et al. 2007;

Sannolo et al. 2014). This opens the possibility of recording large amounts of data while potentially minimising the effect of the observer on the studied system (Langkilde and Shine 2006), possibly resulting in more representative data (Tattersall and Cadena 2010). Additionally, thermography allows gathering high-resolution temperature data (Tattersall et al. 2009), with comparably lower background noise relative to other IR tools (Kastberger and Stachl 2003). Finally, thermography permits more freedom to analyse complex data using available dedicated software, increasing the potential applications of the technology (Sannolo et al. 2014).

The high-resolution temperature data collection capabilities of thermography can also be integrated with the knowledge of the occurrence of regional heterothermy in many reptiles. Since Heath (1964) first described this phenomenon in *Phrynosoma coronatum*, many other groups of reptiles have been shown to demonstrate such capabilities (Sannolo et al. 2014). However, some authors have denied the occurrence of this phenomenon in very small reptiles (Stevenson 1985).

Nonetheless, the onset of thermography has facilitated the further study of this phenomenon in reptile (Bosch 1983; Tattersall and Cadena 2010; Sannolo et al. 2014; Burns et al. 2015) and non-reptile groups (McCafferty et al. 2015; Cadena and Tattersall 2009; Tattersall and Cadena 2010,). Thus, IR tools unlock the possibility of exploring new methods of obtaining comparable temperature data using modern, less intrusive procedures.

This study aims to explore the effects of thermal inertia and regional heterothermy in order to compare the temperature of different body parts of heating and cooling lizards, measured using IR thermal imaging, to their cloacal temperature. Therefore, the aim of this study was ultimately to determine whether it is possible to infer internal body temperature using thermography.

2. Methods

2.1. Study species

This study tested 46 adult male lizards belonging to three different lacertid species, representing three distinct body size-classes observed in European lizards: small (26 *Podarcis virescens*, sensu Geniez et al., 2014), medium (10 *Lacerta schreiberi*) and large (10 *Timon lepidus*). Lizards where noosed from the field in Évora (38.57°N, 7.91°W; *P. virescens*) and Vila do Conde (41.33°N, 8.67°W; *L. schreiberi* and *T. lepidus*) municipalities (Portugal) during spring. Only individuals with intact tails were used for this experiment. The animals were brought to the lab where their snout-vent length (SVL) was recorded to the nearest 0.01 mm using a digital calliper and their weight measured to the nearest 0.0001 g using a precision balance (Sartorius M-Pact AX224, Sartorius AG, Goettingen, Germany).

2.2. Experimental setting

The animals were kept in individual cages with food (*Tenebrio molitor* larvae) and water supplied *ad libitum* and exposed to a natural light cycle regime. During the day air temperature was set at 28 °C, while during the night was set at 20 °C. Within less than seven days, all animals where returned to their respective sites of capture.

All lizards were individually subjected to a thermal gradient (± 20– 50 °C) in an acrylic terrarium (100×30×40 cm) covered with a < 0.5 cm layer of vermiculite acting as a substrate. A 150 W infrared reflector bulb, fixed 25 cm above the substrate at one end of the terrarium was used as the main heat source (Carretero 2012). Ambient air temperature was maintained around 20 °C by an air conditioning system.

To obtain readings from heating and cooling animals, lizards were subjected to 4 h of testing in the gradients with the heat lamp turned on followed by 4 h with the lamp turned off. Every hour, a FLIR T335 thermal camera (sensitivity: < 0.05 °C; accuracy: ± 2%; IR image resolution: 320×240 pixels; Flir Systems Inc., Wilsonville, Oregon, USA) was used to simultaneously take an IR and a regular photo of each lizard's entire body (skin emissivity=0.96). IR camera was hand held and photos were shot at 30–40 cm depending on the subject size. This approach allowed us to maintain always the same resolution in every IR image, irrespective of species and body size. Immediately (< 20 s) after photographing each animal, the subject was captured and its cloacal temperature measured with a contact thermometer (Hibok 18, precision: 0.1 °C, accuracy: ± 0.2%) fitted with a k-type thermocouple probe. The reading was obtained by inserting the probe few millimetres into the cloaca of the animal.

2.3. OLS and RMA regressions

Upon completion of all tests, IR photos were analysed using the software FLIR Tools 2.1 (Copyright 2014 FLIR Systems, Inc; http:// www.flir.com). The *Spotmeter* function of this software was used to measure the temperature at six body locations, as shown in Fig. 4.1.1: snout, eye, head (at the base of the parietal scales), dorsal (centrally), base of tail (dorsally, above the location of the cloaca) and left hind limb (at the knee articulation). When these were not easily identifiable in IR photo, the corresponding normal photograph was used to determine the location of where to obtain the reading from.

Given the non-normality of the data (Shapiro-Wilk tests < 0.05 for all species individually), a Kruskal-Wallis test and Dunn post-hoc tests were used to test the statistical significance of the difference of both the SVL and the weight, between the three species groups.

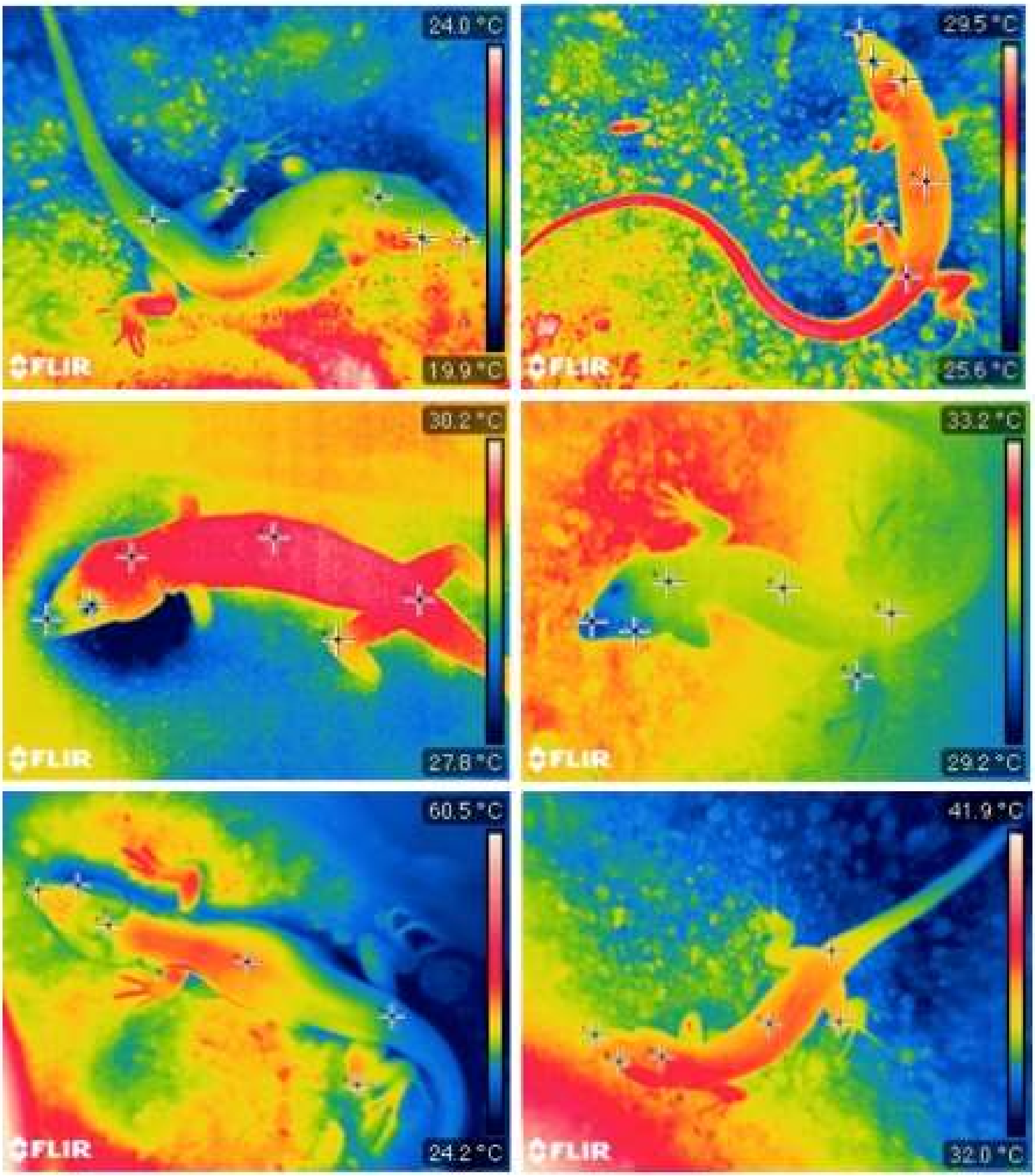

Fig. 4.1.1. IR photographs of *Timon lepidus* (left) and *Lacerta schreiberi* (right), showing thermal heterogeneity, while basking in the thermal gradients. The pictures also serve to show from where temperatures were measured on the lizards' bodies.

Due to the lack of normality of the residuals and given the repeated measures design of the experiment, a Method II regression with resampling was deployed to perform an Ordinary Least Square (OLS) to test for a relationship between cloacal temperature and the temperature of each body part measured using IR imaging (Legendre and Legendre 2012). OLS was also deemed a more appropriate method than Reduced Major Axis (RMA) regression. Cloacal temperature, even though it is measured with a certain degree of error, is being used as a proxy for inferring internal body temperature. In this case, the variables assigned to the X and Y axes are not arbitrary and, hence, the presence or absence of symmetry in the regressions of Y on X and X on Y becomes irrelevant (Smith 2009). Nonetheless, with large correlation coefficients, the slopes of OLS and RMA, should not differ to a large extent anyway (Smith 2009).

2.4. Linear mixed-effects models

We further investigated the relationship between body temperature (from both cloacal and IR readings) and variables that could possibly affect it. Given the unbalance structure of the data and the possible subject-specific effects, we fitted a Linear Mixed-Effects Model. Body temperature was set as the dependent variable and three variables and their interactions as predictors. The set of variables was treatment*- body position*species (where treatment is either heating or cooling) and individuals were treated as random effects. The starting model were reduced following Zuur et al. (2009) and normality of the model's residuals were checked graphically (Pinheiro and Bates 2000).

All the statistical analysis was performed in R (R Development Core Team 2015) using the lmodel2 v1.7-2R package (Legendre 2012) for the resampling procedure, the lme4 package (Bates et al. 2015) for the linear mixed models and the lmerTest package for the Post-hoc tests on the mixed models.

3. Results

3.1. Size categories

Podarcis virescens was the smallest lizard (mean weight=4.41 ± 0.24 SE; mean SVL=57.97 ± 1.55 SE), followed by *Lacerta schreiberi* (mean weight=25.92 ± 1.47 SE; mean SVL=98.61 ± 2.37 SE) and *Timon lepidus* (mean weight=69.05 ± 3.43 SE; mean SVL=137.42 ± 2.77 SE). Kruskal-Wallis rank sum tests and following pairwise Wilcoxon rank sum tests, corrected with False Discovery Rate (FDR), showed that species were different with respect to weight, SVL and body temperature ($P < 0.0001$ for all pairwise comparisons for each variable).

3.2. Cloacal vs. IR readings

Podarcis virescens had an overall lower temperature than the two green lizards during both heating and cooling, a larger range and reduced variability in the temperatures measured (Figs. 2b and 3). The green lizards also showed more similar measured temperatures and ranges between them than with *P. virescens* (Supplementary material).

OLS regressions models, summarized in Table 4.1.1, showed moderate to-good fits (R^2 from 0.838 to 0.968). For all the species, the body location that provided the best statistical fit (highest R^2) was the base of the tail. Yet, as shown by the confidence intervals in Table 4.1.1, only the snout did not show an OLS slope different from 1, despite a considerably lower statistical fit.

The eyes of the two green lizards showed the same relationship, also accompanied by a decrease in the strength of the model. On all other body parts, the slopes of the best-fit lines exceeded 1, thus suggesting that the IR readings increased at a faster rate than those taken from the animals' cloaca.

This effect is displayed in 4.1.3 where the pattern of cloacal temperature change over time is less exaggerated than that of most of the other body part temperatures obtained with the IR camera. IR camera tended to inflate the body temperature readings since in all body parts and for both treatments (except for the eye and leg of *P. virescens*) the lines of the body temperatures, measured with the IR camera, are above the line of the cloacal temperature (Figs. 2c, and 3).

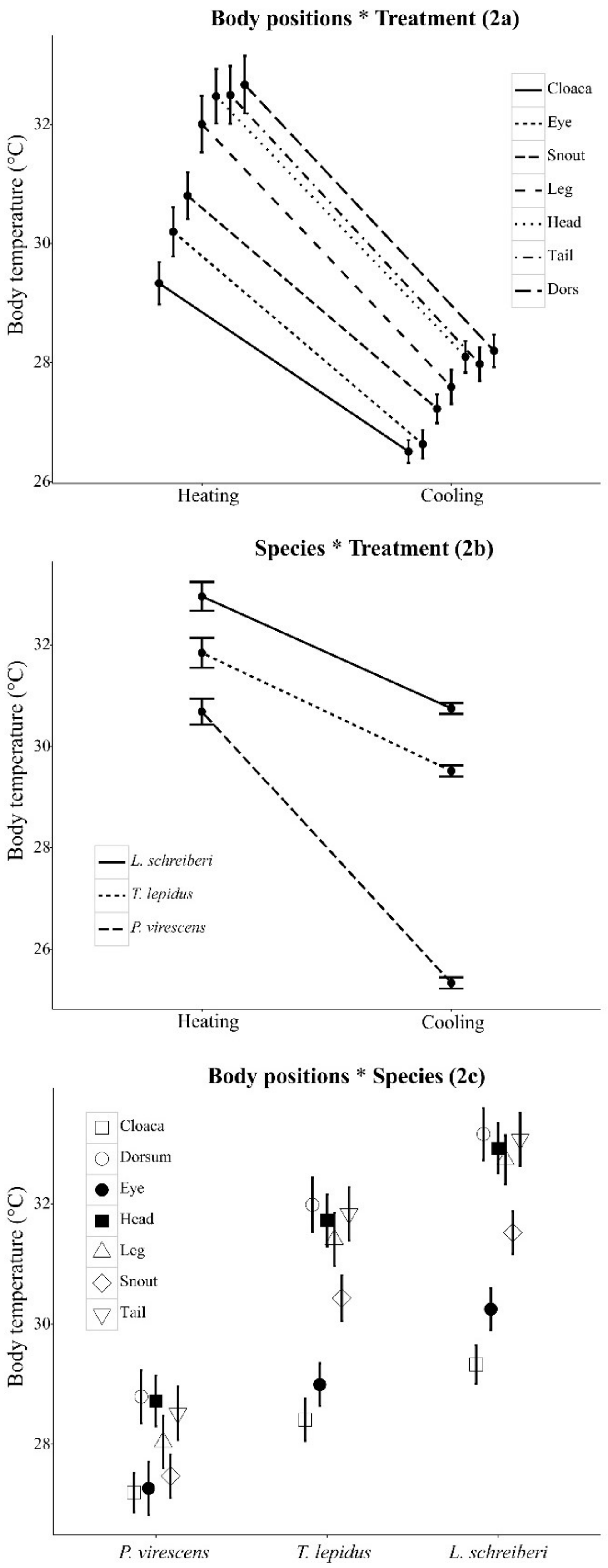

Fig. 4.1.2. Graphs describing the three significant interaction terms of the Linear Mixed Effects models. Top (2a): temperatures for all body positions under heating and cooling conditions. Middle (2b): body temperature of the three species for both treatments. Bottom (2c): temperature of each body position for the three species.

3.3. Linear mixed-effects models

According to the model, the following variables had a significant effect on body temperature: the random effect (P < 0.0001; d.f.=1), and the three interactions treatment*species (P < 0.0001; d.f.=2), body position*species (P=0.0011; d.f.=12), and treatment*body position (P < 0.0137; d.f.=6). Extensive post-hoc analysis is available as Supplementary materials.

Fig. 4.1.2 provides some insight into the pattern of the three significant interactions for the three species. In Fig. 4.1.2a can be appreciated that temperature relationship among body parts was consistent in both treatments (regardless of the species). Fig. 4.1.2b shows that body temperature was actually higher under heating conditions for all species. *P. virescens* showed an abrupt change in body temperature passing from heating to cooling, possibly because of the reduced body size. Finally, in Fig. 4.1.2c it can be observed that the relationship of body temperature with each body position is consistent across species. The dorsum, head, tail and leg reached the highest temperature, followed by the snout and the eye, while cloacal temperatures were consistently the lowest among all body positions.

	Podarcis virescens					*Lacerta schreiberi*					*Timon lepidus*				
	R^2	Slope	Inter-cept	Slope 95% CI's	P-perm	R^2	Slope	Inter-cept	Slope 95% CI's	P-perm	R^2	Slope	Inter-cept	Slope 95% CI's	P-perm
Snout	0.838	0.996	0.28	**0.934** **1.058**	*0.001*	0.884	0.997	2.27	**0.920** **1.073**	*0.001*	0.900	1.038	0.86	**0.964** **1.112**	*0.001*
Eye	0.879	1.087	-2.22	1.023 1.151	*0.001*	0.934	0.972	1.70	**0.917** **1.028**	*0.001*	0.914	0.972	1.31	**0.909** **1.035**	*0.001*
Head	0.910	1.247	-5.18	1.193 1.300	*0.001*	0.955	1.215	-2.68	1.159 1.271	*0.001*	0.961	1.183	-1.91	1.133 1.234	*0.001*
Dorsal	0.916	1.302	-6.63	1.248 1.356	*0.001*	0.956	1.258	-3.70	1.201 1.315	*0.001*	0.952	1.213	-2.50	1.156 1.272	*0.001*
Tail	0.931	1.316	-7.29	1.267 1.366	*0.001*	0.964	1.272	-4.17	1.219 1.324	*0.001*	0.968	1.206	-2.43	1.160 1.252	*0.001*
Leg	0.924	1.298	-7.26	1.246 1.349	*0.001*	0.959	1.194	-2.25	1.141 1.248	*0.001*	0.925	1.155	-1.39	1.084 1.226	*0.001*

Table 4.1.1. Results of the OLS regressions fitted on the models of temperature of body part x (measured with IR camera) ~ cloacal temperature. Underlined values indicating the body part that gives the best fit for each species and bold values indicate where the slope was not significantly different from 1. Italics indicate significant P-perm values for regression slopes.

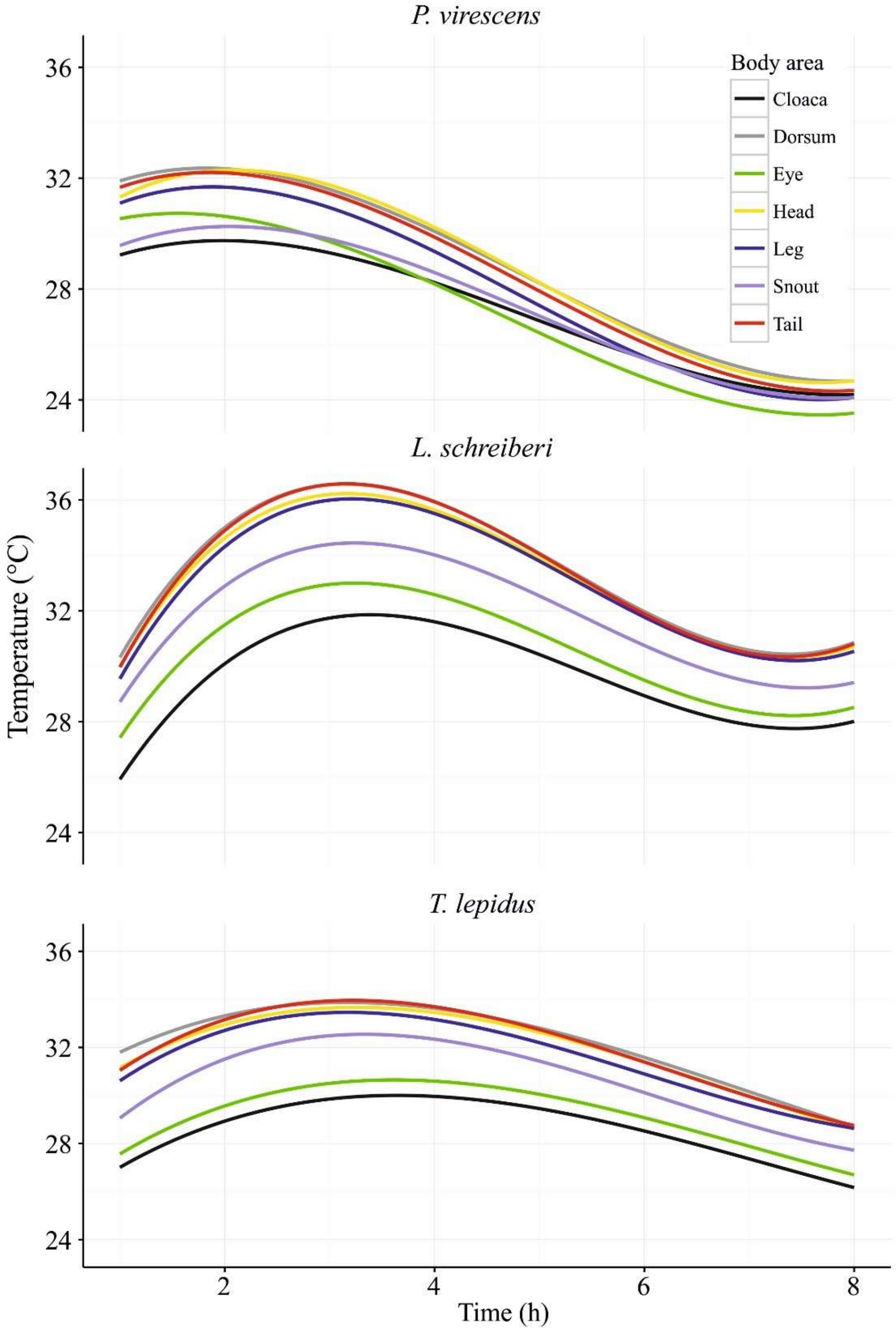

Fig. 4.1.3. Graphs showing the patterns of heat gain (hours 1–4) and loss (hours 5–8) in the different body parts of *Podarcis virescens*, *Lacerta schreiberi* and *Timon lepidus* (top, middle and bottom, respectively) over the course of the experiment. Each colour corresponds to one body area. Lines were smoothed using a third order polynomial function.

4. Discussion

An increasing number of studies use thermography to obtain temperature data from a large variety species (Heath 1964; Bosch 1983; Tattersall and Cadena 2010; Sannolo et al. 2014). Despite this, it seems to be few attempts to validate the use of IR equipment to infer internal body temperature of reptiles by comparing this technology to more traditional methods, like cloacal thermometer (Luna et al. 2013). Most studies, like Carretero (2012) regarding pyrometry, warn towards the uncertainty of using IR technology to measure surface temperature and infer internal temperatures of small-sized ectotherms, cautioning for larger thermal inertia and bigger effects of regional heterothermy and skin-core temperature gradients with increasing body sizes (Jones and Avery 1989). Therefore, the aim of the present work was to assess the employability of infrared thermography in lizard thermal ecology studies and determine the potential of using such tool to infer internal body temperatures.

The results from the present study suggest that measurements taken with an IR camera from several body locations correlate strongly ($R^2 > 0.83$) with cloacal temperature obtained through contact thermometry. Contrastingly, the study by Carretero (2012) found much weaker correlations (R^2=0.308–0.668) between cloacal and dorsal temperature, measured with an IR thermometer in four species of the genus *Podarcis*. The strong correlations found in the current study suggest the presence of a definite relationship between skin and internal temperatures of the lizards. Nonetheless such finding is not novel. It has been widely reported that reptiles are able to regulate body temperature distribution (thus creating core-surface thermal gradients) behaviourally and physiologically by adjusting blood flow between the skin and core, through thermal hysteresis, or by eye bulging (Dzialowski and O'Connor 2001; Bucklin 2006; Tattersall et al. 2006).

The strength of the reported correlations holds for small to large body sized lacertid lizards, albeit the exact pattern seems to be body-part specific, but consistent in all species (Fig. 4.1.2c). This outcome is also supported by the results of the linear mixed-effects model. In fact, we are able to quantify (Figs. 2 and 3, Supplementary material) that the green lizards achieved larger differences between cloacal and skin surface temperatures than the much smaller *P. virescens*. We consider that such effect is most likely attributable to the larger thermal inertia of the green lizards but we cannot discard underlying species-specific physiological and/or behavioural mechanisms being the cause of the observed pattern (Bell 1980; Zamora-Camacho et al. 2014). However, the relationship among the temperature of the different body positions, as well as the effect of cooling and heating, was consistent irrespective of the body size. We thus believe that, at least for the studied species, body size does not represent an impediment to the use of IR thermography to investigate their thermal ecology.

Different body parts correlate differently with cloacal temperature. For instance, the strongest correlations (highest R^2), like the tail, are not the ones that more closely mimic temperature changes in the cloaca. For instance, the eye followed very closely the absolute values of cloacal temperature, followed by the snout, for all species (Fig. 4.1.2c). However, neither the eye nor the snout showed the strongest correlations to cloacal temperature (compare the lines slopes in Fig. 4.1.2a). The remaining body parts (dorsal, tail, head, limb) all experienced temperatures larger than the eye and the cloaca, during both heating and cooling (Fig. 4.1.2c).

This complex outcome could be due to a multitude of reasons, namely the smaller thermal inertia of the head region (Heath 1964; Georges 1979), larger temperature ranges experienced by the snout due to evaporative cooling and variable breathing rates (Bosch 1983; Tattersall et al. 2006), small size of the measured area that made it more susceptible to the pixel size effect (Faye et al. 2016), and the effect of background radiation.

Several studies suggested that reptiles may regulate their head temperature more precisely than the rest of the body by resorting to evaporative cooling from moist surfaces such as the eyes (King and Green 1999) or the mouth and the snout (Heath 1964; Georges 1979; Hillenius 1992; Tattersall et al. 2006). This implies that the tissues surrounding these areas should be highly irrigated with blood coming from the rest of the body (Bruner 1907). These surfaces are therefore very likely to be isothermal with the internal environment of the animal and hence will likely follow the pattern of temperature change in the brain, where the hypothalamus (the temperature regulation centre) is located (Seebacher and Franklin 2005). This wealth of evidence supports our findings that suggest the eye and snout as potentially the best direct proxies for internal body temperatures.

Since the other body points are further away from the hypothalamic environment, they may be expected to experience larger temperature fluctuations (Gordon and Heath 1983). In fact, our results partly support these expectations as we observed overall higher temperatures on the tail, limbs and dorsal skin surfaces than those measured on the eye or snout. Additionally, these areas also experienced increasing ranges of temperature from the basking to the cooling phases.

Interestingly, the temperatures measured on the head (skin surface temperature) closely followed the pattern of the more peripheral body points, which is quite discrepant from the pattern observed in the eye, snout and the cloaca. Such discrepancy most likely relates to the fact that current technology is sensitive to surface temperatures (Goller et al. 2014; Vollmer and Möllmann 2010; Sannolo et al. 2014). The skin therefore is more likely to undergo larger fluctuations in temperature than the internal organs since it is in contact to external thermal environment while the temperature of the internal organs is buffered by the whole animals' thermal inertia (Bakken 1989; Tattersall and Cadena 2010).

We acknowledge that obtaining good quality IR images containing the eye (or the snout) of small animals might be challenging, particularly under field conditions. This emphasizes the need of careful consideration in planning studies with IR thermography. Taking together the results concerning the strong but variable relationship between cloacal temperature and other body parts, the effect of body size and the practical difficulties in obtaining eye temperature in the field, we believe that the best approach for using IR thermography could be represented by a species-specific calibration of the tool under use. Alternatively, data analysis should be carried out on IR picture only, without a direct comparison with other tools (Goller et al. 2014).

Finally, some authors are already suggesting using skin temperature (as measured with IR tools) as a proxy for body temperature in lizards (Berg et al. 2015). This latter approach, however, poses problems in comparing new data obtained with IR tools with the available literature, which is almost entirely based on internal (cloacal) temperature. Finally, IR thermography represents a tool that causes little or no stress to the studied animals. Even though calibrating the device against the study species might be time-consuming, we believe that the advantages can largely compensate for the need of proper calibration. This technology allows collecting more data with a better precision and in less time, if compared with cloacal probes. Likewise, the amount of stress induced to the studied animals during repeated manipulations and insertions of the probes is completely eliminated if contactless tools, like IR thermography, are used.

5. Conclusions

From our study, we conclude that thermography can be an invaluable tool in thermal ecology studies, particularly for those researchers wishing to obtain high-resolution temperature data of several body parts simultaneously and/or continuously (Tattersall and Cadena 2010; Goller et al. 2014; Faye et al. 2016).

We provided evidence to support the use of eye temperature as the best and direct approximation of internal temperature. Otherwise, other body parts can be used, provided that a careful calibration is carried out before inferring internal temperature from IR readings.

Protocols relying on thermography must be meticulously planned with caution not to depart from the assumptions haunting the use of this equipment (i.e. thermal inertia effects, regional heterothermy, thermal hysteresis, skin-core thermal gradients). Finally, future studies should focus on gaining further insight into the potential of this technology applied to thermal ecology studies.

Conflict of interest

There are no conflicts of interest to declare.

Author contributions

FMB, MAC and MS conceived and designed the research; FMB and FS collected the data; FMB and MS performed the analyses; FMB, MAC and MS wrote the manuscript.

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4.2 Article II: Waitin' on a sunny day: factors affecting lizard body temperature while hiding from predators

Journal of Thermal Biology. In Revision

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Abstract

Most animals face predators in their daily life and have evolved antipredator strategies that promote survival while minimizing escaping costs. For example, many animals hide into refuges into which their pursuers cannot access. Ectotherms rely on external sources of heat to raise their body temperature, and it can be expected that they pay a cost in terms of heat loss while staying hidden. Indeed, refuges are often more thermally unsuitable than the external environment. To measure the body temperature of an ectotherm both before and after a predation attempt without interfering with its behavior is challenging. Here we used infrared technology to measure the body temperature of a large-sized lizard (*Timon lepidus*) before individuals escaped from a simulated predation attempt to hide inside a refuge, and after they emerged back from the refuge. We quantified the change of body temperature that lizards experienced while hiding and show that while the decrease in body temperature covaries with the time spent hidden, and it is also affected by the initial body temperature. In contrast, the time spent hidden depends mostly on the temperature inside the refuge. Indeed, lizards hiding in warmer refuges spent more time hidden, likely benefitting from a reduced cooling rate. Thus, lizards perceive and evaluate the thermal quality of their refuges and integrate this information to react to predation attempts and minimize the potential thermal consequences of hiding.

Keywords: Thermal ecology; Refuge use; Thermoregulation; Antipredator behavior; Lacertidae; *Timon lepidus*

1. Introduction

Many animals, both vertebrate and invertebrate, avoid predation by entering in temporarily refuges small enough to prevent their pursuers to fit in (Frix et al., 1991; MacHutchon and Harestad, 1990). Several studies investigated hiding strategies, both theoretically (Hugie, 2003) and experimentally (Jennions et al., 2003). Optimality models predict that antipredator behavior allows animals to increase their fitness through survival and future chances to reproduce (Cooper and Frederick, 2007a; Cooper and Frederick, 2007b). Various models predicting hiding time have been theorized and tested (Johansson and Englund, 1995; Polo et al., 2005).

Hiding in refuges usually entails costs as well as benefits. By hiding, animals often cannot feed, thus exchanging survival in the short term against the loss of energetic income (Koivula et al., 1995). The loss of feeding opportunities depends on the relative balance of predation risk and food availability in the environment (Martín, López, and Cooper, 2003b). Likewise, while hiding an individual cannot engage in social activities like mating, thus losing opportunities to reproduce (Kålås et al., 1995). Other costs involve the risk of intrusion of competitors into the animal's territory (Díaz-Uriarte, 1999) or the exposure to a different predator while hiding (Soluk, 1993). Some of these costs may also vary depending on the sex or the season (Brown and Shine, 2004).

The physiological cost of hiding, especially regarding heat loss, may be particularly relevant for ectotherm species, since they rely on external sources of heat to raise their body temperature and keep it close to a preferred and often narrow value (Angilletta, 2009; Huey, 1982). Hiding lizards may experience suboptimal conditions inside their refuges (Wolf and Kramer, 1987) since air temperature is usually lower than their preferred one (Martín and López, 1999b). Therefore, the body temperature of hiding lizards may drop below the range at which several physiological functions are optimized, like locomotion (Angilletta et al., 2002), digestion (Van Damme et al. 1991), and prey-handling (Avery and Mynott, 1990). Furthermore, when emerging from a refuge, lizards may be forced to spend time thermoregulating to recover their original body temperature and lose opportunities to feed (Downes 2001) and be potentially exposed again to predators (Martín and López 2001). Hence, the maintenance of the preferred body temperature is critical in many lizard species, as suboptimal body temperatures can lead to a decrease in performance and, in general, in fitness (Angilletta 2009). The reliance on external sources of heat, coupled with the suboptimal thermal conditions encountered inside refuges make lizards an excellent model to study hiding strategies. Indeed, a vast wealth of knowledge is now available on optimal hiding time (Polo et al., 2005), on the effect of body size (Martín and López, 2010), on intrasexual competition (Díaz-Uriarte, 1999) and on the impact of multiple types of predators (Stapley, 2004). A recent compendium on antipredator behavior (Cooper and Blumstein 2015) also summarized the hiding strategies of lizards (Martín and López, 2015).

Unfortunately, to our knowledge, no study measured the decrease in body temperature that lizards may experience while hiding from a predator under natural conditions. Indeed, a recent review of antipredator behaviors stressed that methodological difficulties prevented so far to study the relationship between body temperature and escaping behavior (Cooper, 2015). Such challenges arise from the need to record the body temperature of an animal both before and after a predation attempt, in order to calculate the resulting difference due to hiding. A common practice in field research on lizards is to capture an individual only after a predation attempt, and measure its cloacal temperature with a thermocouple, thus getting a single measure of body temperature. Several studies recognized this methodological limit and inferred the thermal costs of hiding indirectly, for example using air or operative temperature (Cooper and Wilson, 2008; Martín and López, 1999a, 1999b). Quantifying to what extent lizards' body temperature change while hiding may be thus of critical importance to develop more accurate models of optimal hiding time and understand how behavior and physiology interplay in wild ranging ectotherms (Polo et al., 2005).

To overcome these practical difficulties, we used Infrared Thermography (IRT), that measures the infrared radiation emitted by an object and allows to quantify an animal's surface body temperature from a distance, without capturing the individual of interest (Speakman and Ward, 1998). The steady development of cheaper and portable thermal cameras provides nowadays the opportunity to investigate several aspects of animal biology. IRT is currently used to study veterinary injuries (Kastberger and Stachl, 2003), to estimate animal densities (Gill et al., 1997), as well as in human physiology (Ring and Ammer, 2012). In ectotherm studies, IRT provided insights on physiology (Sannolo et al., 2014), ecology (Goller et al., 2014) and behavior (Pérez et al., 2016).

In the present study, we used IRT to measure the change in body temperature that lizard experienced while hiding from a predator and to study the environmental and biological factors that may influence heat loss and hiding time. We predicted that (1) lizard body temperatures fall below their preferred body temperature while hiding, due to the unsuitable thermal conditions inside the refuges; (2) lizards body temperature decreased proportionally to the time spent inside refuges; that (3) bigger lizards lose heat at a slower pace than smaller ones, due to their higher thermal inertia; and that (4) the thermal quality of the refuges will affect the duration of hiding, with warmer refuges inducing a prolonged retreat.

2. Methods

2.1. Study Site

The present study took place during 2016 at Castro São Paio (Labruge), a coastal area of approximately 5 ha in Northern Portugal (41° 16’ 54” N; 08° 43’ 50” W). The area is characterized by a mixture of small agricultural corn fields, grassland, and bush patches (mainly Ulex, Erica and Carpobrotus species), while trees are almost absent. Low-height dry stone walls (50-150 cm) and rocky outcrops are abundant, delimiting walking paths and fields. Data collection occurred in September, a period during which Ocellated lizards are not sexually active and dedicate most of their time to feeding and thermoregulation (Mateo and Castanet, 1994). By focusing on the last part of the active season, we were able to exclude confounding factors like sexual competition, body condition, and egg-bearing-induced thermal shifts in female lizards (Carretero et al., 2005).

2.2. Model Species

The Ocellated lizard, *Timon lepidus* (Fig. 4.2.1) is the biggest European lizard, with a snout-vent length (SVL) that grow up to 240 mm in S Iberia, but only up to 195 mm in NW Iberia (Mateo and Castroviejo, 1991). It is a territorial and thermophilic species, usually associated with bushes and large rocky outcrops (Díaz et al., 2006). It is often preyed by large raptors (Martin and Lopez, 1996), snakes (Mateo, 2014) and occasionally carnivorous mammals (Padial et al., 2002). In the study area, Ocellated lizards live close to dry stone walls and rocky outcrops, inside which they seek refuge if pursued. The preferred body temperature of the species has been estimated using both IRT and cloacal readings in a previous work, in which the body temperature of various body parts were measured with IRT while animals were free to move in a thermal gradient (Barroso et al., 2016). In a thermal gradient, the preferred temperature of adult males is ~32 °C. In the field the body temperature of this species in the southern part of its distribution range is comprised between 21.2 and 34.5 °C (Busack and Visnaw, 1989). We discarded juveniles (SVL < 100 mm) due to the practical difficulties in measuring their body temperature accurately from a distance. Individuals between 100 and 125 mm were considered subadults. These features make the Ocellated lizard an excellent model to test for the effect of predatory attempts on hiding strategies and body temperature.

2.3. Data collection

Opportunistic sampling was carried on from approximately 9:00 to 19:00 h, covering the daily time span of activity of the Ocellated lizard. Upon detecting a lizard with binoculars, one of us (MS) approached it slowly and took a thermal picture from ~3 m with a FLIR T335 thermal camera (Fig. 4.2.1: sensitivity: < 0.05 °C; accuracy: ± 2% of the reading; IR image resolution: 320×240 pixels, field of view 25° × 19°, lens 25 °; Flir Systems Inc., Wilsonville, Oregon, USA). The same researcher then simulated a predation attempt by further approaching frontally the lizard at a constant speed to induce an escaping reaction. To avoid confounding effects that may affect risk perception of lizards (Cooper et al., 2003), the same person wearing the same cloths performed all approaches. Given its large body dimension, *Timon lepidus* seeks refuge into big crevices of the dry-stone wall or under large rocks (Díaz et al., 2006). As soon as the lizards hid, we started a stopwatch and moved away to observe from a distance (8-10 m) the entry of the refuge. Following the procedure of Martín and López (2010), we measured the time it took for the lizard to show the head first (appearance time) and the time for full-body emergence (recovery time, from the beginning of hiding).

As soon as the lizard fully emerged from the refuge it was approached again, a second thermal picture was taken, and then it was captured by noosing. We measured the SVL (snout-vent length; to the nearest mm with a digital caliper) and head length (to the nearest 0.1 mm). We also measured the air temperature and the relative humidity inside the refuge (Mini-Hygrometer Mod. GBC KTI-903; temperature precision ± 0.5 °C, humidity precision ± 2.5 %), as well as the height from the ground of the refuge entrance (nearest cm). To avoid pseudoreplication, we took dorsal, ventral and lateral pictures of each individual and crossed photo-identification with biometric variables to ensure that each lizard was tested only once (Sacchi et al., 2016).

2.4. Thermal images analysis

We inspected each thermal image with the software FLIR Tools 2.1 and extracted temperatures using the Spotmeter tool (Copyright 2014 FLIR Systems, Inc; http://www.flir.com). For each picture, we extracted the head and trunk temperature, since in lacertid lizards these two areas may be at a different temperature even in a controlled environment (Sannolo et al., 2014). A recent study demonstrated that the readings from both the head and trunk provide a good proxy for Ocellated lizards' internal temperature, at least under laboratory conditions (Barroso et al., 2016). We thus calculated the decrease of head and trunk temperature as the difference between the thermal picture shot before approaching the lizard and the one shot after the appearance of the head and the rest of the body, respectively.

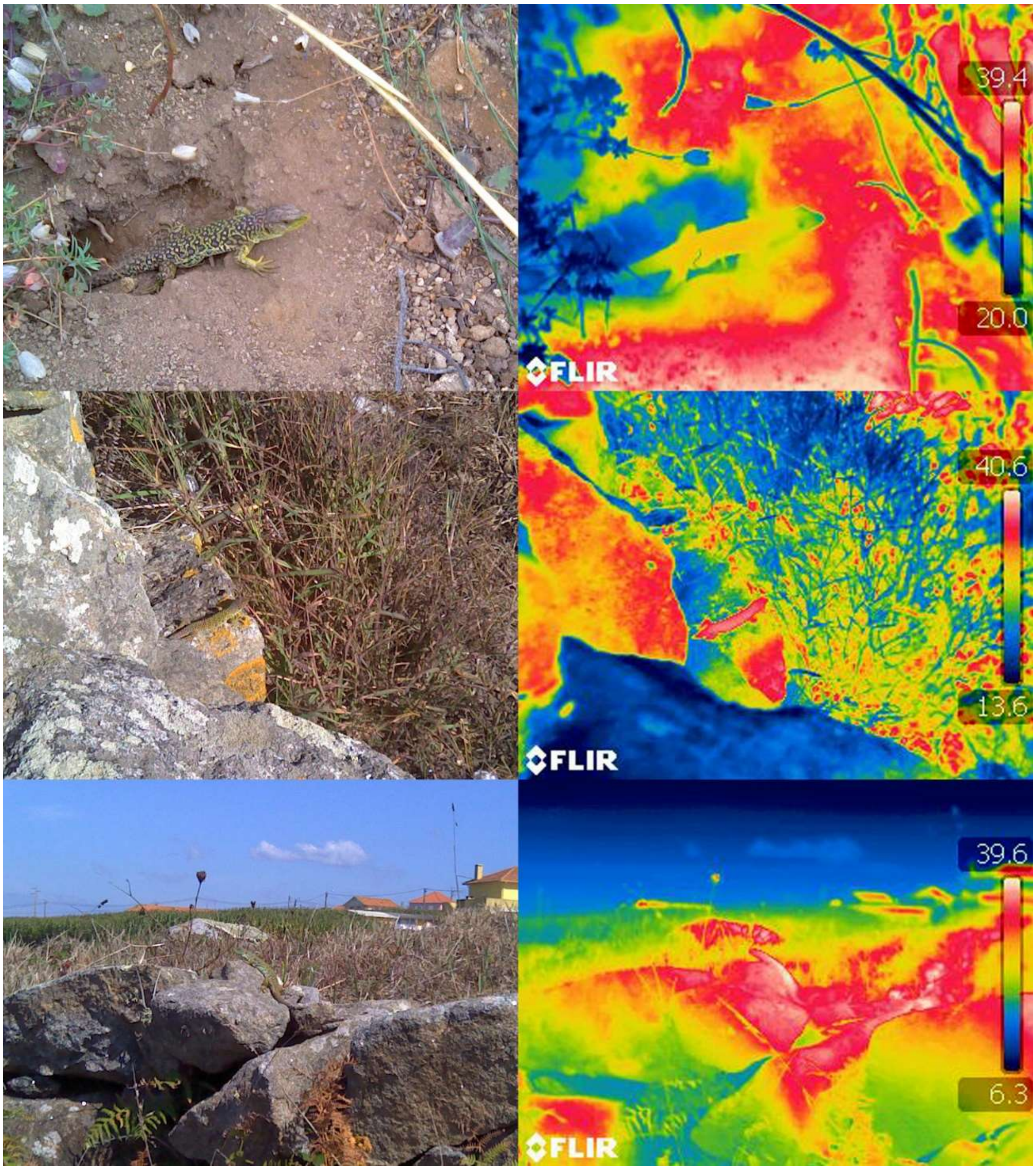

Fig. 4.2.1. Examples of the thermal and visible images taken during the study and used to extract body temperatures. The temperature scale on the right margin is expressed in Celsius degrees.

2.2. Statistical analysis

We computed descriptive statistics for the change in temperature in the head and trunk, as well as for the time spent hidden until appearance and recovery time. We tested for difference between head and trunk temperature both before and after hiding since the only evidence for head-trunk temperature difference in lacertid lizards come from laboratory observations (Barroso et al., 2016; Sannolo et al., 2014). We compared the head and trunk temperature after hiding with published data on the preferred temperature for the same population (Barroso et al. 2016), to estimate to what extent lizards are willing to drop their body temperature below their preferred one. We used structural equation modeling (SEM) to assess the relationship between appearance and recovery time and several predictors (Lomax and Schumaker, 2004). The starting model for appearance time included as predictors the decrease in body temperature, the refuge temperature, the relative humidity inside the refuge, the initial head temperature, the head length and the height from the ground of the refuge. A second model was fitted for trunk temperature. Some predictors were the same, but SVL replaced head length, initial trunk temperature replaced the initial head temperature, and the decrease in body temperature replaced the decrease in head temperature. Potential multicollinearity was checked both qualitatively through pairwise correlations plots and quantitatively through VIF analysis. VIF values < 3 were considered as not correlated (Hair et al. 2006). Because SEM cannot deal with unordered factors, the potential contribution of sex and age on hiding behavior was assessed separately using ANOVA.

All statistics were performed in the R environment (R Core Team, 2018). SEM models were fitted using the package lavaan (Rosseel, 2012). Figures were produced with the package ggplot2 (Wickham, 2009). Unless otherwise specified, results are expressed as mean ± standard deviation (SD).

3. Results

3.1. Time spent hiding, cooling, and preferred temperature

We collected full data for 56 lizards (20 males, mean SVL = 142 ± 17 mm; 19 females, mean SVL = 130 ± 10 mm; 17 subadults, mean SVL = 113 ± 6 mm). Appearance time ranged between 19 and 435 s (118 ± 96 s), while recovery time ranged between 29 and 613 s (156 ± 125 s). Head temperature before hiding was 30.97 ± 2.63 °C, while trunk temperature was 32.16 ± 3.11 °C. Such head-trunk temperature difference before hiding was statistically significant (Paired Student's t-test = -5.8474, P < 0.0001, d.f. = 55). After hiding, head temperature dropped to 29.82 ± 2.51 °C, while trunk temperature dropped to 30.46 ± 2.86 °C. The head-trunk temperature difference remained significant also after hiding (t-test = -3.4422, P = 0.0005, d.f. = 55). The difference in temperature before and after hiding was significant for both the appearance and recovery time (t-test = 8.6262, P < 0.0001, d.f. = 55; t-test = 10.414, P < 0.0001, d.f. = 55, respectively). After hiding, lizards lost 1.15 ± 1.04 °C in the head and 1.69 ± 1.27 °C in the trunk. The greatest decrease in head temperature was 3.6 °C, measured in an adult female after hiding for 370 s. Instead, the greatest decrease in trunk temperature was 5.3 °C, measured in a subadult that hid for 415 s. The mean rate of temperature decrease was 0.58 °C/min for the head and 0.65 °C/min for the trunk. Refuge temperature ranged between 18 and 27.6 °C (mean 23.3 ± 2.3)

While both the head and trunk temperatures of lizards were, on average, close to the preferred one selected in thermal gradients before escaping (Barroso et al. 2016), they dropped, on average, approximately 2 °C below the preferred ones when emerging from the refuge.

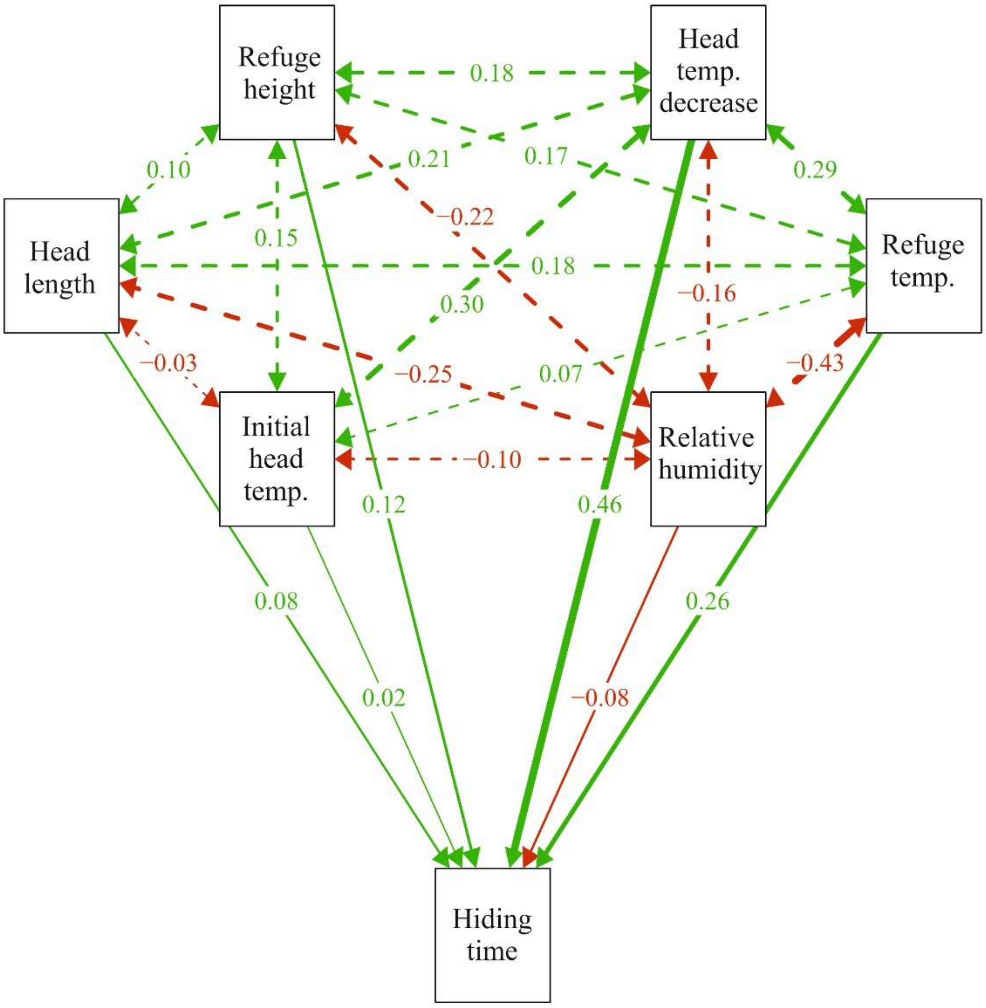

Fig. 4.2.2. Path analysis diagram showing the relationship among the outcome variable (emergence time, bottom) and predictors. Note that the relationships among predictors are expressed as correlations coefficients, with the double-headed arrows implying not causal relationship. Instead, the relationships between the outcome and predictors are expressed by the path coefficients, which are standardized versions of linear regression weights.

3.2. Predictors for appearance and recovery time

The SEM model for the head accounted for 47.3% of the variance in appearance time, while the mode for recovery time accounted for the 43.7% of the variation. The results of SEM analysis (Fig. 4.2.2 and Fig. 4.2.3) showed that hiding time and heat loss covaried (appearance time: z-value = 4.233, P < 0.0001; recovery time: z = 2.314, P = 0.021), but was modulated by refuge quality, with lizards hiding in warmer refuges waiting more before emerging (appearance time: z-value = 4.233, $P < 0.0001$; recovery time: z = 2.311, P = 0.021). The other predictors considered, like SVL, humidity, initial temperature and refuge height did not influence hiding time ($P > 0.05$ in all cases). Among the predictors, although all were only weakly correlated (Fig. S1 and Fig. S2 for all pairwise comparisons), the strongest relationships were between refuge humidity and temperature (-0.43) and between the heat loss and the initial head (0.30) or trunk temperature (0.37).

We found no statistical differences in hiding time among sexes ($F_{1,37} = 2.132$, P = 0.153). Similarly, adult lizards were not associated with refuges of higher thermal quality than subadults ($F_{1,54} = 0.265$, P = 0.609).

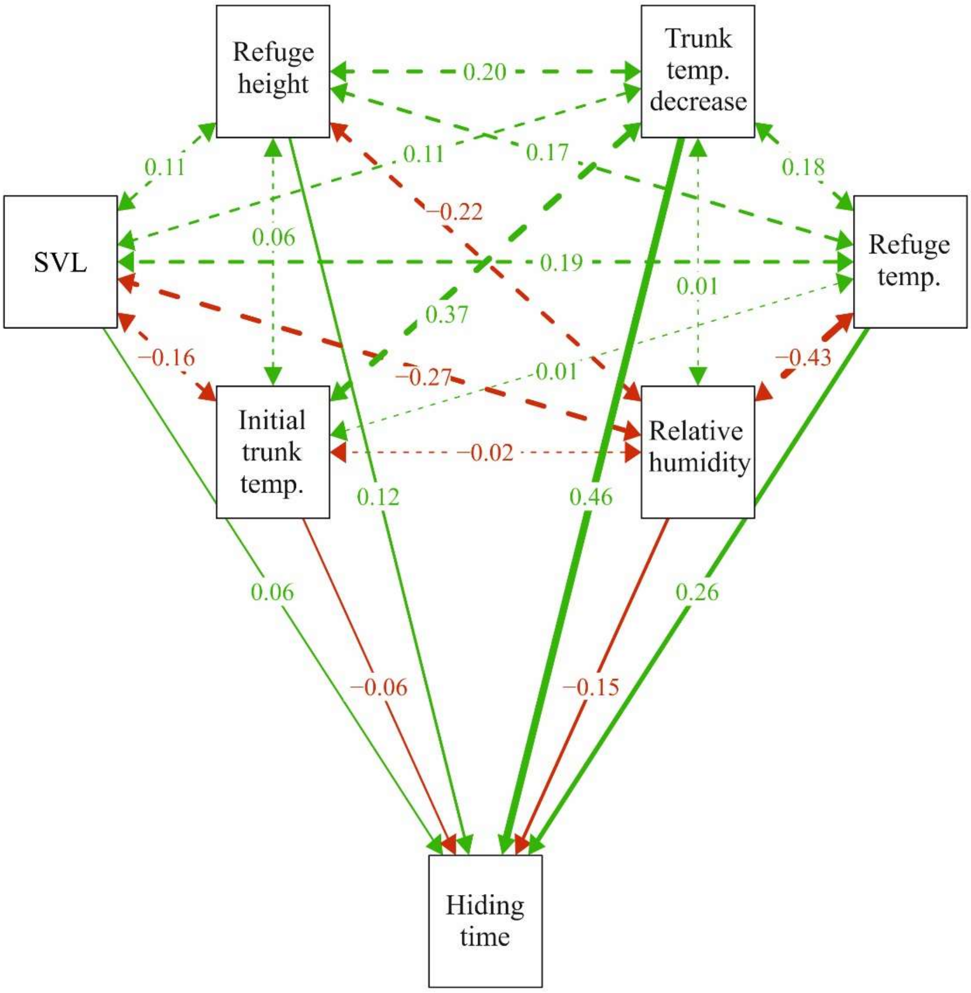

Fig. 4.2.3. Path analysis diagram showing the relationship among the outcome variable (recovery time, bottom) and predictors. Note that the relationships among predictors are expressed as correlations coefficients, with the double-headed arrows implying not causal relationship. Instead, the relationships between the outcome and predictors are expressed by the path coefficients, which are standardized versions of linear regression weights.

4. Discussion

We provide here the first field quantification of the decrease in body temperature that free-ranging lizards may experience as a consequence of hiding from a predator. Of the initial four hypotheses, we found statistical support for three of them. We showed that after hiding lizards' body temperature dropped below their preferred one; that such a decrease was proportional to the time spent hidden and that the thermal quality of the refuge affected the recovery behavior. Instead, we found no support for the potential effect of body size, likely due to the reduced variability of body size of our sample, composed solely by adult lizards.

Cooling of both the head (Fig. 4.2.2) and the trunk (Fig. 4.2.3) covaried with hiding time. This result was the most expected since several studies already quantified the cooling rates over time for various lizard sizes under laboratory settings (Dzialowski and O'Connor, 2001; Kour and Hutchison, 1970). However, we present here novel quantifications for wild ranging lizards. In our sample, the duration of hiding, and hence the resulting decrease of body temperature were, on average, modest. Indeed, most lizards spent from a few seconds up to seven minutes before showing the head, while lizards waited no more than 10 minutes before repositioning at their original position. Nonetheless, some lizards lost over 5 °C of their initial body temperature, indicating that in some cases hiding behavior may potentially entail high thermal costs. Such costs would be represented by the time needed to restore the original body temperature, which would depend on environmental conditions (radiation intensity, air temperature and wind speed), and by potential missed opportunity to feed (Downes 2001). The decrease in body temperature was mainly associated with the initial temperature either of the head or trunk, with warmer individuals losing more heat. However, warmer individuals did not spend more time hidden, nor were those individuals hiding in cooler refuges. The apparent contradiction is explained when considering that cooling does not vary linearly with the difference of air temperature inside the refuge, but instead, it follows Newton's law on cooling (Maurone and Shiomos, 1983).

Both appearance and recovery times correlated with the thermal quality of the refuges, with lizards hiding in warmer refuges waiting more before showing the head and the rest of the body. In the present case, lizards' body temperatures were close to the preferred ones before escaping. Conversely, when recovering from hiding, temperatures dropped approximately 2 °C below the preferred one for this species, that could have significant consequences on lizards. Indeed, lizards are able to use information on refuge quality in the context of antipredator behavior (Polo et al., 2005; Andersson et al., 2010), while in the field they show complex hiding strategies depending on the thermal quality of the refuge (Martín and López, 2000; Cooper and Wilson, 2008). Warmer refuges may slow the cooling rate of a lizard, thus directly reducing the thermal consequences of hiding from a predator. Hence, an ideal refuge would have a temperature close to the preferred by the lizard and entails no thermal cost (Huey and Slatkin, 1976). Therefore, if the temperature is viewed as a resource (Magnuson et al. 1979), it is likely that lizards compete for refuges of higher thermal quality, if they are limited. Competition may vary depending on population densities, predator pressure, external operative temperature, and refuge availability (Downes and Shine, 1998).

We predicted that lizard's size would affect the cooling rate, but body length correlated neither with hiding time nor with the decrease in body temperature. It should be considered that having focused only adult lizards, we inevitably sampled only a small portion of the overall variability in body size for this species. Indeed, other animal groups showed a size-related variation in antipredator behavior (Krause et al., 1998; Sih, 1992). Different behavioral, social, and thermal pressures on large and small individuals may explain our results as well. Larger individuals may choose refuges not only based on their thermal quality but also looking for protection against predators or as possible nesting sites (Kerr et al., 2003). On the other hand, juvenile lizards may be more cautious due to their higher residual reproductive value (Clark, 1994), and refuge selection may be based mainly on their thermal properties. Similarly, due to their smaller dimensions, juveniles may choose a broader variety of refuges. However, juveniles were not measured in this study, thus reducing the range of variation in body size. Moreover, as already discussed, hiding time was generally short, possibly contributing to mask the effect of size. Finally, we simulated only a single predator attack. Multiple predation attempts may force lizards to hide for prolonged periods or force variation in activity pattern and antipredator behavior, potentially boosting heat loss (Martín et al., 2009).

Our results showed that sex did not influence hiding time, even though other lizard species have proved to vary in their hiding time depending on sex, at least during the reproductive season (Martín, López, and Cooper, 2003a). By choosing the post-reproductive season, we likely removed sex-related confounding factors, like egg and sperm development. Heliothermic lizards living in temperate areas like the Ocellated lizards devote autumn mostly to optimising fat accumulation before winter for the subsequent breeding season, through eating and thermoregulating as accurately as possible (Carretero, 2006). Hence, intersexual differences that may otherwise play a role during the reproductive season were absent in our study. Furthermore, to estimate how the time in the refuge, throughout a season, would decrease fat accumulation, it would be needed a prolonged field study with observations over time to estimate how often lizards retreated in response to disturbance in a season. Such a research would be of interest, especially in conservation and management in areas with intense human, cat, and dog activity.

Relative humidity and the height from the ground of the refuge did not influence hiding time. Relative humidity was generally high in all refuges (range 50-88 %, mean 65 %) and likely the rate of heat loss inside the refuges was not affected by this variable. Instead, humidity may play a relevant role in nocturnal or wintering refuges (Galán, 1999). Height from the ground may influence the internal structure and thermal quality of refuges, as well as to affect the cooling rate and vary the exposure of lizards to predators and competitors (Bakken, 1989). Indeed, it can be hypothesized that increasing height may correlate with air flow, light intensity and inversely with humidity. Even though in this study Ocellated lizards used refuges evenly from the ground to approximately 150 cm, it is challenging to ascertain the actual position of a lizard inside the wall. A more specific study on refuge quality and its physical characteristics (height, internal structure, light, temperature and humidity) may add meaningful data on the subject.

Finally, the results obtained in the present study were made possible by exploiting the non-invasiveness of IRT technology. By shooting thermal pictures both before and after a predation attempt, we were able to quantify heat loss without affecting lizards' behavior. The same results cannot be obtained by more traditional methods, like measuring the cloacal temperature, that requires the capture of the individual of interest. Hence, IRT is opening vast opportunities to study in the field several aspects of ectotherm ecology that have eluded previous investigations (Goller et al., 2014; Sannolo et al., 2014). However, our approach bears limitations as well. Indeed, a few lizards (4) showed negative values of heat loss for the head, suggesting that they gained some heat while hiding. Instead, this likely results from the observer being forced to wait for each lizard to fully emerge before shooting the second thermal picture, leading to underestimating heat loss from the head. Lizards that gained heat are those with longer vigilance time that lead their head to heat again before exiting with the rest of the body ("head basking", see for example Heath 1964; Gregory 1990). Hence, the trunk may represent a more accurate proxy than the head for measuring the thermal consequences of hiding behavior in those species that routinely patrol with the head before showing the rest of the body. Nonetheless, the lizards' head represents a fundamental component for thermoregulation, being the physiological thermostat (Cabanac et al., 1967). Hence, it is challenging to assess if lizards regulate more strictly the head or trunk temperature. Such a challenge is further confounded by the behavioral role (patrolling) that the emerging head often has in lizards.

5. Conclusions

By measuring lizard's temperature both before and after a standardized predation attempt, we have been able to directly quantify the resulting heat loss, resulting from a predation attempt. Even though previous studies attempted to infer the thermal costs of hiding indirectly (Martín and López, 2010), as far as we know this is the first quantification of the thermal consequences of hiding behavior in lizards. Even though the average decrease in body temperature was modest regarding absolute values, it varied considerably among individuals and led to suboptimal body temperatures for this species (Barroso et al., 2016). Hiding time correlated with the decrease in body temperature and was modulated by the thermal quality of the refuges. Therefore, lizards hiding from a predator are likely forced to thermoregulate to regain the heat lost. However, basking may expose lizards to the old predator or a new one, or competitors (Huey and Slatkin, 1976). Hiding lizards are thus forced to balance potential thermal costs against predator pressure. It may be hypothesised that depending on predator and competitor pressure and refuge quality, lizards may show variable hiding times (Polo et al., 2005). Future works should focus on how the variability in the thermal quality of the refuges may affect antipredator behavior and how this is modified by other pressures such reproduction, predation intensity or competition (Frix et al., 1991; Hedrick and Kortet, 2006).

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Chapter 5: Thermal ecology, water balance and performance of two closely related lacertid lizards

"Competitor species can have evolutionary effects on each other that result in ecological character displacement"

— Grant and Grant (2006)

5.1 Article III: Physiological differences in preferred temperatures and evaporative water loss rates in two sympatric lacertid species

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Abstract

Sister species living in sympatry offer the opportunity to study the degree of divergence in their ecological, physiological and life-history traits. It has been hypothesized that closely related species with overlapping distribution should differ in their niche to reduce competition for resources. Furthermore, the investigation of sympatric species may shed light on how they may coexist without outcompeting each other. In the present study, we assess the degree of physiological divergence in two sympatric lacertid lizards, *Podarcis bocagei* and *Podarcis guadarramae lusitanicus*. These species share a Pliocenic ancestry and overlap at a both geographical and ecological scale. We assessed their thermal preferences and water loss rates, two physiological traits considered stable across congeneric species. We found that the two species differ in both traits, with *P. bocagei* selecting higher temperature than *P. g. lusitanicus* and losing more water than the latter at and above its preferred temperature. The results also showed that for both species body size has a relevant impact on thermal and hydric traits, with bigger individuals losing proportionally less water and selecting higher temperatures. These results, combined with previous evidence, suggest that physiological mechanisms, ecological preferences and morphology probably allow these two species to overlap in their distribution while selecting different microhabitats and thus decreasing possible competition between them.

Keywords: Lacertidae, *Podarcis*, Sister species, Thermal ecology, Water balance, Preferred temperature

1. Introduction

Closely related species with sympatric distribution offer the opportunity to study the extent of divergence in their ecology, physiology and life-history traits (Connell 1983). It has been hypothesized that closely related species belonging to the same ecological guild and living in sympatry should differ to reduce competition (Schulter 1994). Alternatively, specific combinations, or assemblages of species are expected to exclude one another (Diamond 1975).

Patterns of divergence in sympatric species are common and encompass a large variety of taxa. For example, interspecific competition and competitive exclusion appear to be relevant in shaping ant communities (Andersen and Patel 1994), and niche partitioning might be a powerful force segregating fish species in terms of ecological and morphological space (Wikramanayake 1990). In amphibians, the degree of character displacement can be associated with the intensity of competition between tadpoles of closely related species sharing the same ponds (Pfennig and Murphy 2002). Depending on the case, either genetic or non-genetic changes can lead to reduced competition through niche shift associated with phenotypic plasticity or character displacement (Pfennig and Murphy 2002; Price et al. 2003).

Lizards represent an excellent model to study adaptation, competition, niche shift and habitat segregation. For example, *Anolis* lizards from the Caribbean islands have been intensively investigated in this regard on each island, either one or more *Anolis* species are present, with different species assemblages on each island (Roughgarden 1995). If several species live on the same island, they segregate into different ecomorphs that evolved several times independently (Losos 1990). It has also been shown that sympatric lizards differ in scale morphology, likely the result of adaptation to different climatic niches (Wegener et al. 2014). Thus, in *Anolis* lizards, interspecific competition has driven the evolution of morphology, behavior, and physiology, shaping the species composition on each island to reduce niche overlap among species (Losos 1994).

Lizards are also excellent model organisms to investigate thermal adaptation, with seminal studies dating back to the first decades of the previous century (Bogert 1949). An overwhelming amount of evidence has been collected regarding the thermal ecology of many species, as well as the relationship between environmental temperature, body temperature and fitness (Cossins and Bowler 1987; Angilletta 2009). Ectotherms rely on external sources of heat to raise their body temperature and many of them actively thermoregulate (Angilletta 2009). One of the most important themes in thermal biology is related to the concept of the preferred temperature (T_{pref}) that represents a range of temperatures, usually narrow, within which most physiological functions are performed nearly at their maximum (Huey and Stevenson 1979). For lizards, it has been hypothesized that T_{pref} should be either an evolutionary labile or static trait, depending on the view and the history of the considered group. The labile hypothesis focuses more on local adaptation, while the static one stresses the evolutionary rigidity of T_{pref}, considering it an umbrella parameter for many physiological functions (Hertz et al. 1983). On the other hand, thermal ecology is strongly intertwined with water balance. For instance, it has been shown that dehydrated lizards tend to prefer lower body temperature (Crowley 1987). However, for a long time most studies on lizards focused solely on the thermal aspect of their ecology.

The current state of knowledge on water balance, evaporative water loss (EWL) rates and the effect of temperature on the hydric status of lizards is poor, particularly if compared with the amount of studies focused solely on lizards' thermal biology. For example, in desert dwelling lizards, differences in EWL were shown to be attributable to habitat selection (Dmi'el 2001), while in *Anolis* lizards it has been found that species living in drier and warmer climates tend to have fewer dorsal scales, probably to reduce cutaneous water loss (Wegener et al. 2014). However, there is surprisingly little evidence regarding physiological differences among sympatric lizards, especially from temperate climates. García-Muñoz and Carretero (2013), for example, showed that *Algyroides marchi* and *Podarcis hispanica* select similar temperatures but are characterized by different water loss rates, possibly associated with different microhabitat selection. To our knowledge, EWL has never been studied at various temperatures for sympatric sister taxa of lizards.

Podarcis wall lizards have been used as model species to investigate many aspects of reptile biology, from evolution (Harris and Arnold 1999; Herrel et al. 2008) to morphology and life-history traits (Bauwens and Diaz-Uriarte 1997; Kaliontzopoulou et al. 2012). Conversely, the thermal and hydric ecology of *Podarcis* species has been investigated in some species but little is known on the relation between thermal ecology and water balance, with studies usually focusing on the thermal preferences of a single species (Ji and Braña 1999). Regarding EWL rates, information is even more scarce, with data available only for a few species, showing that, in general, either congeneric or heterogeneric species living in sympatry differ in their relative EWL (GarcíaMuñoz and Carretero 2013; Osojnik et al. 2013; Carneiro et al. 2015). *Podarcis* species of the Iberomaghrebian clade are an intriguing case to study ecophysiology, due to their high level of genetic diversity coupled with a complex pattern of evolutionary and biogeographical history (Carretero 2008; Kaliontzopoulou et al. 2011).

In the present study, we examined these two ecophysiological traits, T_{pref} and EWL, using two sister taxa of lacertid lizards, *Podarcis bocagei* and *P. guadarramae lusitanicus* (*sensu* Geniez et al., 2014). These two species were once included into the *Podarcis* *hispanica* species complex, from which several lineages have been elevated to species or subspecies level (Harris and Sá-Sousa 2002). Most of the forms in the complex show an allopatric or parapatric distribution, while *P. bocagei* and *P. g. lusitanicus* are largely sympatric in their distribution range in northwestern Iberian Peninsula (Kaliontzopoulou et al. 2011; Geniez et al. 2014). These two species are characterized by one of the closest genetic distances among the recognized species pairs within the complex (Kaliontzopoulou et al. 2011). In contrast with this phylogenetic proximity, recent studies highlighted how these two closely related species differ substantially in morphological traits and microhabitat use (Kaliontzopoulou et al. 2012; Gomes et al. 2016). In particular, *P. bocagei* is more robust and seems associated with a more ground dwelling life style in comparison with *P. g. lusitanicus*, which has a more compressed head, associated with a more saxicolous life-style. Furthermore, the former is restricted to Atlantic climate, while the latter occurs both in Atlantic and Mediterranean areas (Loureiro et al. 2008). This mixture of phylogenetic affinity, coupled with overlap in their distribution and divergence in microhabitat use and morphology, makes this two-species system a good candidate to investigate the existence of a divergence in physiological traits directly linked to thermal and hydric biology.

In this context, we expected that (i) if the labile view of T_{pref} is found in the study system, the two species should differ in thermal preferences due to current or past adaptive processes, even though they are sister taxa; (ii) if instead one of the two species selects for higher temperatures, it should also lose more water, assuming a trade-off between thermal preference and the rate of evaporative water loss,; (iii) considering species distribution, climate affinity, and microhabitat use, *P. g. lusitanicus* would be more resistant to water loss than *P. bocagei*.

2. Methods

2.1. Individual sampling and housing

Lizards were collected by noosing near Moledo, a coastal locality in northern Portugal (41°50 N, 8°52 W) where the two species live in syntopy. To avoid the effects of reproduction, body condition and ontogeny on T_{pref} and EWL, only males with intact tails were captured (Carretero et al. 2005). Samplings took place during August and September 2015. To avoid recaptures across sampling sessions, we applied photo-identification using I^3S Classic (Van Tienhoven et al. 2007; Sacchi et al. 2016; download at http://www.reijns.com/i3s/). Lizards were brought to the lab where their snout-vent length (SVL) and head height (HH) were measured to the nearest 0.01 mm using a digital calliper and their body mass was measured to the nearest 0.0001 g using a precision balance (Sartorius M-Pact AX224; Sartorius AG, Goettingen, Germany). All measurements were taken by the same person (MS). Lizards were housed in individual terraria (40 cm × 25 cm × 25 cm) for two days before experimentation. During this period, lizards had ad libitum access to water and shelter, but no food was given to prevent digestion from affecting T_{pref} and EWL (Van Damme et al. 1991). During captivity, lizards were exposed to a natural light cycle regime with daytime air temperature set at 27 °C, and nighttime air temperature set at 20 °C. By the end of the experiment, all lizards were released at the point of capture.

2.2. Preferred temperature

We individually exposed 37 *P. bocagei* and 32 *P. g. lusitanicus* to thermal gradients (± 20–50 °C) by putting them in 100 cm × 30 cm × 40 cm acrylic terraria provided with a 150 W infrared lamp at one end. A thin layer of sand (~5 mm) acted as a substrate. Room temperature was kept at 20 °C by an air conditioning system to guarantee the establishment of the gradient inside the terraria. Individuals were granted 24 h of habituation to experimental conditions before the experiment started. Then, every hour, for nine repetitions (10:30-18:30), we measured cloacal temperature with a contact thermometer (Hibok 18, precision: 0.1 °C) fitted with a k-type thermocouple probe. We limited each contact with lizards to 10–15 s to prevent them from changing body temperature during the readings. This procedure might cause some stress to the lizards, but we believe it represented the best option, considering that the use of infrared thermometers or trailing thermocouples attached to the cloaca is not feasible on small species (Trullas et al. 2007; Carretero 2012). To prevent the lizards from losing water during the experiment, possibly altering the pattern of T_{pref} (Crowley 1987), a cup of water was provided in the middle of each gradient. By the end of the experiment, lizards were returned to their original terraria. We calculated the mean temperature (± standard deviation), the temperature distribution and the 50% central range as a proxy for thermal preference (Hertz et al. 1993). A two samples t-test was used to compare thermal preferences of the two species, while F-statistics from linear models were used to assess the effect of SVL and body mass on thermal preferences.

2.3. Evaporative water loss rates

The same individuals tested for T_{pref} were tested for EWL rates after one day of recovery with water available. Three temperatures were set for the experiment: 27, 32 and 37 °C. These temperatures were chosen after a small sample of *P. bocagei* (10 adult males, different from the ones tested here) preferred temperatures of approximately 32 °C (using the same procedure as described in Section 2.2). We thus used this value as the reference temperature and then added and subtracted 5 °C. Notice that lizards were kept at 27 °C during captivity. This temperature thus should provoke less pronounced hydric stress, while 32 and 37 °C were expected to elicit increasingly higher water loss. We randomized the order at which every individual was tested with respect to temperature treatment and allowed a one-day recovery after each test. With this design, the lizards' body masses did not statistically change from the beginning to the end of the experiment ($F_{2,204} = 0.216$, $P > 0.80$).

Before the EWL test, lizards were weighed to the nearest 0.0001 g and then put into individual PVC cylindrical boxes (10 cm × 9 cm) with ventilation holes on the top and bottom sides. Groups of 6 individual boxes were placed into a sealed incubator (Binder KB 53, precision at 25 and 40 °C: ± 0.2 and 0.3 °C, respectively) with 125 g of silica gel. This amount of silica gel provided stable and dry conditions (< 15% RH) inside the incubator throughout the experiment. Every hour, for 9 hours, we removed and weighed each individual with the same balance.

Faeces account for a small, but possibly relevant portion of lizards' body mass. Following earlier study methodologies (Carneiro et al. 2015), we did not remove faeces when we found them in the plastic boxes. Even though some lizard species are able to survive upon losing 30% of their body mass in water (Hertz 1980), our aim was to test for differences between species and not for overall resistance to water loss. We thus ceased the experiment if an individual exceeded 5% of body mass loss (Table S5 in the supplementary online Appendix).

Fig. 5.1.1. Two similar-length individuals of *Podarcis guadarramae lusitanicus* (top) and *P. bocagei* (bottom). Notice the difference between these two species in terms of their relative body height and bulkiness.

2.4. EWL data analysis

Given the unbalanced and time-dependent structure of the data and the possible subject-specific effects, we fitted a linear mixed-effects model (Pinheiro and Bates 2000). Water loss was the dependent variable and three variables, and their interactions were the predictors. Considering that the species differed in body mass (see Section 3.1) and were thus likely to lose water in function of their body mass, we used percentage of water loss instead of total water loss. These two variables are highly correlated (Pearson's correlation coefficient: $r_p = 0.93$) and by using percentage we could directly test for species divergence in water loss. The set of explanatory variables was temperature × -species × time (where temperature was either 27, 32 or 37 °C; this set was meant to mimic a realistic thermal scenario that lizards could face in nature). Considering that water loss could vary in a non-linear way through time, we also fit the same model with a quadratic term on the time factor. Body length and body mass were added as covariates to account for potential effects of body size on EWL. Individuals were treated as random effects. The starting model was reduced following Zuur et al. (2009) and normality of the model's residuals was checked graphically (Pinheiro and Bates 2000).

The statistical analysis was performed in R version 3.3.2 (R Core Team 2016) using the nlme package (Pinheiro et al. 2017) for the linear mixed models. Figures were produced using the package ggplot2 (Wickham 2009).

3. Results

3.1. Species morphology

The two species did not differ significantly in SVL (*P. bocagei*: 54.51 ± 5.35 mm, *P. g. lusitanicus*: 53.44 ± 4.52 mm; two sample t-test = 1.01, P = 0.31, d.f. = 67), but they were statistically different in their HH (*P. bocagei*: 5.89 ± 0.59 mm, *P. g. lusitanicus*: 5.30 ± 0.59 mm; two sample t-test = 4.42, P = 0.0001, d.f. = 67) and body mass (*P. bocagei*: 3.75 ± 0.78 g, *P. g. lusitanicus*: 3.29 ± 0.73 g; two sample t-test = 2.55, P = 0.013, d.f. = 66.6). This translates in adult males of *P. bocagei* being more robust, heavier and with higher heads than *P. g. lusitanicus*, while the overall length of the body is similar for the two species (Fig. 5.1.1).

3.2. Preferred temperature

The two species selected statistically different temperatures (two sample t-test = 2.92, P < 0.01, d.f. = 604). *P. bocagei* preferred warmer temperatures in comparison with *P. g. lusitanicus* (31.1 ± 0.13 °C and 30.6 ± 0.11 °C, respectively). Mean difference between the two species was 0.50 °C and it was also reflected in the temperature distributions (Fig. 5.1.2a and Fig. S1 in the supplementary online Appendix). Such a small absolute difference is associated with a small effect size (Cohen's d = 0.23; Cohen, 1992). T_{pref} does not appear to show a clear time-trend pattern (Fig. S2). Following Hertz et al. (1993), the ranges of the preferred temperature (50% central distribution of the data) were very similar between species: 29.8—32.6 °C for *P. bocagei* and 29.4—32.0 °C for *P. g. lusitanicus*. For both species, T_{pref} was positively correlated with SVL and body mass, with bigger and heavier individuals selecting higher temperatures (SVL: $F_{1,591} = 18.97$, $P < 0.0001$; Body mass: $F_{1,591} = 40.58$, $P < 0.0001$; Fig. 5.1.2b).

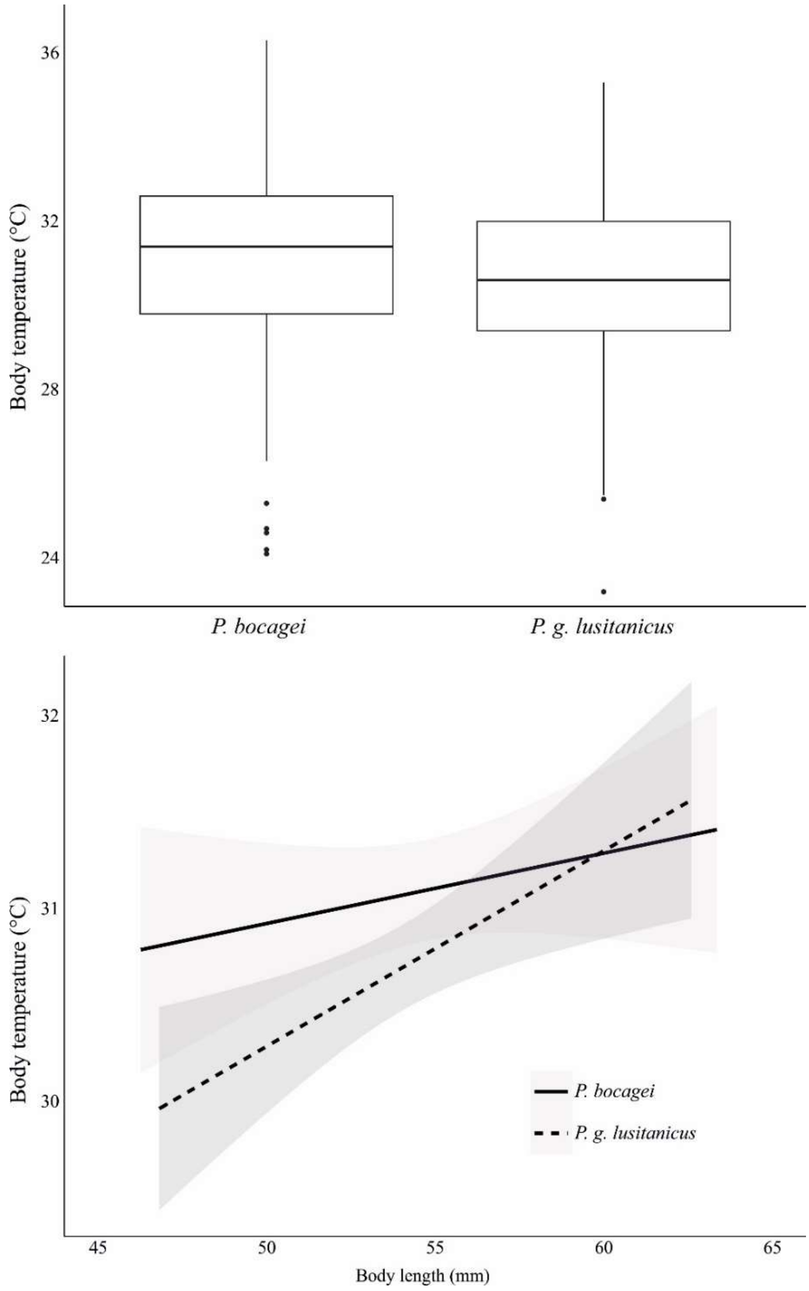

Fig. 5.1.2. Preferred temperature of *Podarcis bocagei* and *P. g. lusitanicus*. Top: boxplot of the temperature distributions for both species. See the text for mean and 50% values. Bottom: linear regression of temperature on the body length. Shaded areas represent the 95% confidence interval.

3.3. Evaporative water loss rates

The quadratic model described the data better than the linear one (Log-Likelihood-χ^2 (LR-χ^2) = 162, d.f. = 22/14, $P < 0.0001$). This suggests that the EWL trend was non-linear with respect to time (Fig. 5.1.3A). After reduction, the best model included the two covariates (SVL and body mass) and the three interaction terms species × - temperature, time × temperature and time × species.

Water loss was negatively correlated with SVL for both species (LR-χ^2 = 27.57, d.f. = 22/17, $P < 0.0001$; Fig. 5.1.3B). Similarly, increasing body mass led to reducing proportionally the loss of water (LR-χ^2 = 22.22, d.f. = 22/17, $P < 0.0001$). Thus, *P. bocagei* appeared to lose more water (in terms of absolute values) if compared with *P. g. lusitanicus* for small individuals, while larger individuals lost proportionally less water.

The significance of the interaction term species × temperature (LR-χ^2 = 17.71, d.f. = 22/16, $P < 0.0001$) reflects the fact that, depending on the treatment, the two species lost different amounts of water. In particular, by the end of the experiment, lizards diverged clearly at both 32 and 37 °C, with *P. bocagei* losing more water than *P. g. lusitanicus* (Fig. 5.1.3C). The pattern is even clearer if loss in terms of absolute values is considered (Fig. S5). Table 5.1.1 shows average amounts of water loss for each temperature treatment, in terms of both absolute values and percentages. The interaction term time × temperature reflects the fact that different temperatures imposed increasing rates of water loss (Fig. 5.1.3D). Finally, the interaction term time × species indicates that the species differed in the overall loss of water across time (Fig. 5.1.3A), regardless of the treatment.

	P. bocagei		***P. g. lusitanicus***	
	g	%	g	%
27°C	0.080	2.220	0.071	2.116
32°C	0.120	3.268	0.096	2.888
37°C	0.154	4.113	0.116	3.693

Table 5.1.1. Average water loss in terms of mass (g) and percentage (%) for both species at each temperature treatment.

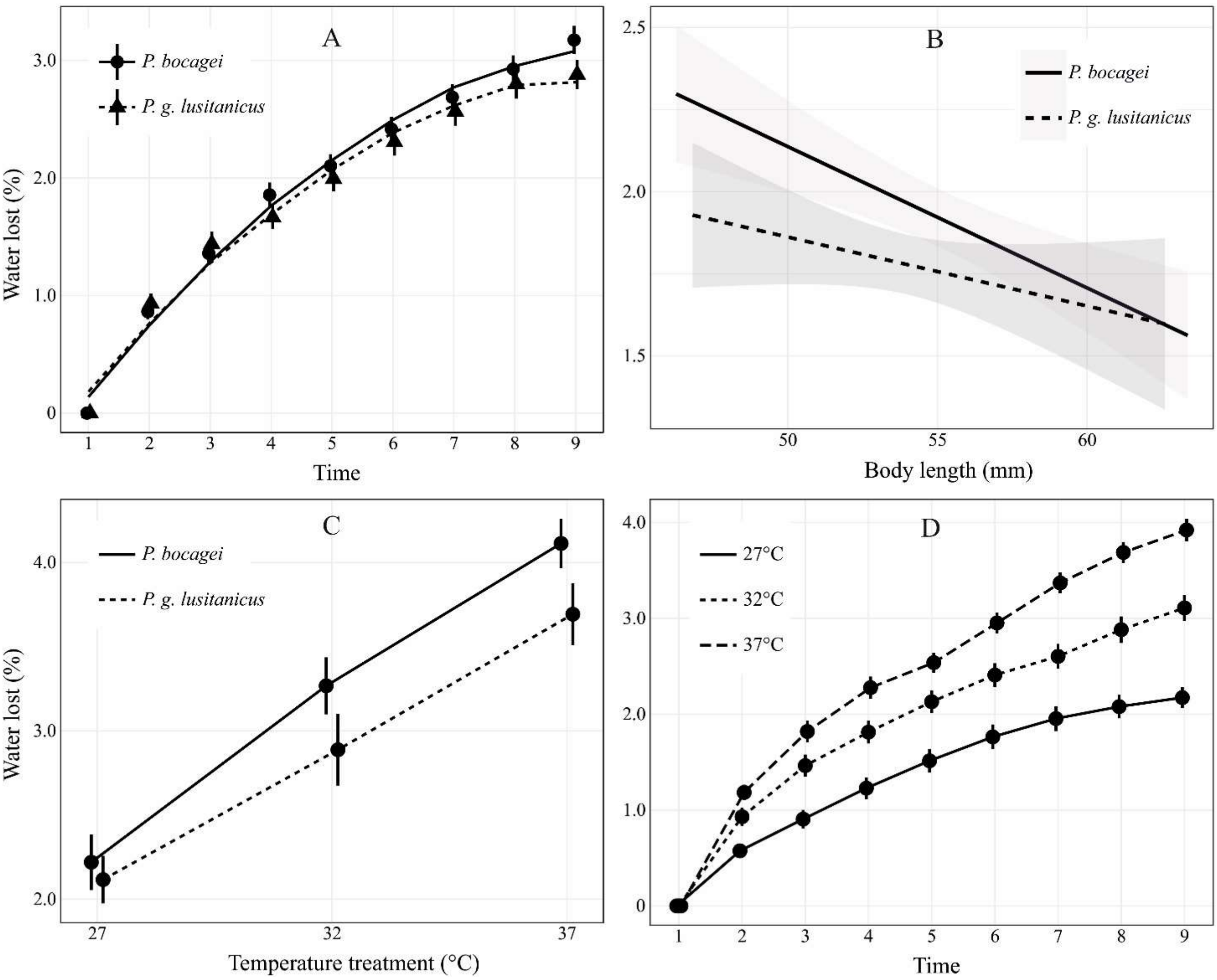

Fig. 5.1.3. (A) Evaporative water loss rate (EWL) as percentage over time, for both species at all temperature treatments. Dots represent measurement data with standard errors, lines represent the fit of the best linear mixed model (see the text for parameterization). (B) EWL as a function of body length for both species. (C) EWL at the three temperature treatments for both species. (D) EWL as a function of time separated for each temperature treatment.

4. Discussion

The results of this study suggest that *P. bocagei* and *P. g. lusitanicus* differ in thermal and hydric physiology. Regarding the thermal preferences, we detected a significant difference, with *P. bocagei* selecting higher temperatures than *P. g. lusitanicus*. In general, higher body temperatures are beneficial to lizards (Angilletta et al. 2010), allowing them to increase their fitness in terms of gametes production (Licht, 1971), digestion efficiency (Harlow et al. 1976), predation avoidance (Christian and Tracy 1981) and locomotion (Bauwens et al. 1995), among others. Thermal preference has been assumed to be either an evolutionarily stable trait, or a labile one, depending on the evidence at hand (Hertz et al. 1983). For example, limited difference has been seen in the thermal preferences of 13 lacertid species (Bauwens et al. 1995). On the other hand, other groups of lizards showed variation in thermal preferences depending on climate (Rodríguez-Serrano et al. 2009) and habitat (Hertz et al. 2013).

The results of our thermal experiment show a relatively small divergence in thermal preference of *P. bocagei* and *P. g. lusitanicus*, considering that they are sister taxa with a relatively recent evolutionary divergence (Pinho et al. 2008; Kaliontzopoulou et al. 2011) and with large geographic overlap (Geniez et al. 2014). In the present study, the absolute difference of T_{pref} between the species was indeed relatively small in terms of absolute values. It is thus likely that from a thermal ecology perspective, the investigated species are an example of evolutionary rigidity for this trait (Hertz et al. 1983). Nonetheless, the differences we detected could still bear an ecological meaning, considering that niche divergence can occur quickly in nature (Pearman et al. 2008), that thermal preference is possibly associated with genetic variation (Good 1993), and that selective pressure to diverge is expected in closely related species living in sympatry (Schulter 1994). For instance, we found that *P. bocagei* selected higher temperatures than *P. g. lusitanicus*. However, the latter is commonly associated with drier and warmer micro-climates, and this pattern seems to hold also at the micro-habitat selection level (Gomes et al. 2016). Further, the geographic distribution of *P. bocagei* is restricted to Atlantic areas (cooler and wetter) while *P. g. lusitanicus* occurs in both Atlantic and Mediterranean areas (warmer and drier; Loureiro et al. 2008). Thus, the biological consequences of the divergence in T_{pref} should be investigated under field conditions and using multiple populations, to understand how thermal preferences relate with other ecological factors and species distribution.

The results of the EWL experiment are clear and could offer an ecological and a physiological framework to understand the direction of the divergence in T_{pref}. For both species, bigger individuals lost less water than smaller ones, suggesting that surface-to-volume ratio is an important factor that contributes to EWL rate even for small body size lizard species (Hertz 1980; Neilson 2002). In absolute values, *P. g. lusitanicus* lost less water than *P. bocagei* at every temperature, and the pattern is similar if percentages are considered: increasingly higher temperatures amplify the difference between the two species. In terms of proximate causes, within-genus divergence in EWL rate can be attributed to peripheral vasomotor changes, involving vasodilation and vasoconstriction of the skin blood vessels (Eynan and Dmi'el 1993).

It is extremely interesting to note that *P. g. lusitanicus* lost less water in proportion to *P. bocagei*, despite being more flattened and lighter, thus having a higher surface-to-volume ratio than the latter (Fig. 5.1.1). These observations, coupled with the fact that the experimental setting prevented individuals from behavioral adjustment, support the hypothesis that the difference in EWL rate between these species can be attributed to physiological mechanisms, allowing *P. g. lusitanicus* to lose less water than *P. bocagei*, despite its higher surface/volume ratio.

Anatomical and physiological differences could not only explain the divergence between species, but also account for the habituation curve in response to temperature. The curvilinear time-trend in EWL appears to be determined by a rapid decline in hour-by-hour loss of water (Fig. 5.1.3A). This pattern likely reflects a stress-induced physiological response that ultimately allowed lizards to reduce their water loss through time by habituation to the experimental temperature (Eynan and Dmi'el 1993). Alternatively, by losing water through time, lizards had progressively less water to lose in relation to their body mass. However, lizards can be quite resistant to water loss. Some tropical species can tolerate up to 30% of body mass loss (Hertz 1980) and some desert-dwelling species can resist up to 50% of loss in body mass (Munsey 1972). In the present study, we limited maximum water loss at 5% of body mass. *Podarcis* species live in temperate, Mediterranean, and continental climate areas (Sindaco and Jeremčenko 2008), and the upper limit of 37 °C (in terms of perceived temperature) that we set was meant to mimic a realistic thermal scenario that these species could face when active during the hottest months of the year. Moreover, our experiment was designed to mimic a diel cycle during which individuals had little access to water, with the aim of testing physiological differences between sister species living in the same area. It is thus possible that *Podarcis* species could tolerate a much higher amount of water loss (Munsey 1972; Hertz 1980).

From an ecological perspective, it is intriguing that the investigated species preferred a slightly different temperature, while losing different amounts of water at their T_{pref}. While *P. g. lusitanicus* prefers a lower temperature than *P. bocagei*, it also loses less water at this temperature, suggesting the existence of a trade-off linking T_{pref} and EWL. Such a trade-off might impose higher water loss rates in species preferring higher temperatures. The contrasting pattern of physiological divergence offers a testable hypothesis under field conditions. It can be expected that, due to its higher EWL resistance, *P. g. lusitanicus* could choose drier micro-habitats than *P. bocagei*. Such micro-habitats might often coincide with higher and windier spots that boost water and heat loss (Bakken 1989). Indeed, field evidence in the site of lizard collection suggests that in these species differences in morphology are associated with micro-habitat selection (Gomes et al. 2016). Similar patterns have been observed in other lizards. For example, in *Anolis cristellatus* xeric-dwelling individuals are more resistant to water loss than mesic-dwelling ones (Gunderson et al. 2011). More in general, EWL rates seem negatively correlated with habitat aridity (Perry et al. 2000) and altitude (Caldwell et al. 2015).

Finally, the results of this study highlight that lizards lose water in function of temperature and that such change is not linear, as reflected by the divergence in the slopes of water loss across time. Therefore, studies testing EWL in lizards should carefully select the temperature at which such assessment takes place. A very low temperature, like 27 °C used here (or lower), could fail to detect patterns of interest between species. For example, Carneiro et al. (2015) did not detect differences between males of two sympatric species of *Podarcis* lizards that were tested for EWL at ~24 °C. This temperature is very unlikely to be biologically meaningful for these species, as the reported T_{pref} for *P. muralis* is 31.76 °C, and for *P. liolepis* it is 31.97 °C, as assessed in the same paper of Carneiro et al. (2015). We thus recommend assessing thermal preference before testing for water loss and then using T_{pref} as a reference temperature for EWL assessment. This protocol offers a meaningful estimate of the amount of water that a lizard is losing at its preferred temperature and allows for a realistic comparison between species. Alternatively, if the main aim is to understand how EWL changes with temperature, it might be useful to measure EWL at various temperatures. Such a protocol would resemble the common one used to estimate performance curves (Angilletta 2006).

Concluding, our experiment highlighted that sister taxa species of lizards living in sympatry might differ in both thermal and hydric physiology. In the present case, *P. g. lusitanicus* selected lower temperatures than *P. bocagei*, and lost less water than the latter, especially at its preferred and higher temperatures. Previous findings suggest that, within a lizard genus, physiological and anatomical differences, in terms of peripheral blood flow and skin properties, could offer a proximate explanation for the observed results. In terms of causal factors, evolutionary divergence in sympatric sister taxa in terms of microhabitat and micro-climate selection could have boosted such physiological differences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.zool.2017.12.003.

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5.2 Article IV: Is suboptimal optimal? A test of Jensen's inequality using wall lizards

In Preparation

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Abstract

Body temperature affects all fundamental aspects of ectotherms biology, like fitness and performance. Ectotherms often regulate precisely their body temperature using behavioral and physiological mechanisms. Thermoregulation is used to maximize whole-organism performance and fitness and it is often assumed that if performance is directly linked to fitness, then natural selection should promote the coadaptation of preferred body temperature and the thermal optimum for physiological performance. Here we used two species of closely related species of Iberian wall lizards to test if thermal sensitivity of sprint speed, a widely accepted measure of whole-organism performance, differs in closely related sympatric species. Furthermore, we tested if the preferred temperatures selected in thermal gradients matched the optimum for sprinting or, instead, if it exists a mismatch between the thermal preference and running performance. We found little difference in running performance between the investigated species, despite they differ in morphology, physiology and habitat use. Hence, running performance seems conservative between this species pair. We also found that running performance are largely unaffected by body temperature, and lizard can sprint nearly maximally over a broad range of body temperatures. Finally, the body temperatures selected in thermal gradients encompass the range of maximum performance, discarding the hypothesis of a mismatch between the thermal preference and the optimum for running. Hence, the tested species are conservative in their thermal physiology possibly suggesting the existence of whole-organism constrains that limit the potential divergence. Finally, the insensitiveness of running performance to body temperature likely allows these two lacertid lizards to perform well in nature under variable temperature conditions

Keywords: Performance curve; Thermal reaction norm; Preferred body temperature; Coadptation; Thermal biology; Lacertidae; Podarcis

1. Introduction

Body temperature affects all fundamental aspects of ectotherms biology, like fitness and performance (Angilletta 2009). Ectotherms mainly regulate their body temperature using behavioral adjustments (Stevenson 1985), physiological mechanisms, and by modulating daily and seasonal activity (Adolph and Porter 1993). Ectotherms typically change their body temperature by exposing to or retreating from direct or indirect solar radiation (Gates 1980), and in many species body temperature is often kept within often narrow species-specific ranges (Cowles and Bogert 1944; Cossins and Bowler 1987). Thermoregulation is hence used to maximize performance (Angilletta et al. 2002) and fitness (Andrews and Schwarzkopf 2012), by achieving and maintaining optimal temperatures for prey handling (Avery and Mynott 1990), digestion (Van Damme et al. 1991), locomotion (Angilletta et al. 2002), reproduction output (Van Damme et al. 1992) and predator avoidance (Christian and Tracy 1981).

The physiological significance and the adaptative role of preferred body temperature (T_{pref}), usually estimated in laboratory thermal gradients (Licht et al. 1966), has been a central argument for decades. Indeed, it is often assumed that if performance is directly linked to fitness, natural selection should promote the coadaptation of preferred body temperature (T_{pref}) and the thermal optimum (To) for physiological performance (Huey and Bennett 1987; Angilletta et al. 2002, 2006). Reptiles and lizards, in particular, have been among the most extensively used models to study both thermal preferences as well as thermal reaction norms and the potential coadaptation of these parameters (Angilletta et al. 2002; Angilletta, 2009). Locomotor performance is the most commonly used proxy for fitness in lizard studies and is often measured as the maximum sprinting speed (Husak 2006). The available literature indicates that, usually, lizards select body temperatures that maximize running performance (Huey and Bennett 1987; Bauwens et al. 1995). However, the optimum temperature for performance curves often exceeds the T_{pref}, due to the non-symmetrical physiological costs of skewed performance curves, a principle known as Jensen's inequality (Martin and Huey 2008). Furthermore, locomotor performance in lizards is generally rather insensitive to body temperature, especially if compared with physiological functions like enzymes activity. Hence, to what extent T_{pref} matches T_o often depends on the species considered and its phylogenetic context.

For some groups of lizards, like Lacertidae, despite the abundant literature available on most aspects of their biology, including thermal ecology (Arnold 2004; Sindaco and Jeremčenko 2008 Corti et al. 2010; Salvador 2014), the information on the potential match between T_{pref} and T_o remains scarce, with many species lacking any research on this aspect. For a few lacertid species, for example, it has been found that those species that reach the highest maximum speeds can also run at near-maximum levels over a wide range of temperatures and maintain body temperatures within a narrow zone near the optimal temperature for sprinting (Bauwens et al. 1995). Here we focus on Podarcis wall lizards, which have been used extensively as models to study physiology, morphology, behavior and thermal ecology. Here we used *Podarcis bocagei* and *P. guadarramae lusitanicus* as a case study. These two sister species are particularly interesting from an ecophysiological perspective because, unlike most other Podarcis species living in the Iberian Peninsula, their distribution range overlap extensively, both at local and wide geographical scale (Carretero 2008; Kaliontzopoulou et al. 2011). Moreover, they differ in their morphology (Kaliontzopoulou et al. 2012), habitat use (Gomes et al. 2016) and thermal ecology (Sannolo et al. 2018). Their common and recent ancestry coupled with differences in morphology and physiology makes these two species an excellent case study to study the potential match between thermal preferences and running performance.

Considering the above-mentioned evidence, we make some predictions on the relationship between thermal preferences and running performance of the species considered here. (1) Being closely related species that live in sympatry, the thermal sensitivity of their locomotory performance should differ, with species-specific optima. Such a prediction is based on the assumption that character displacement, in the form of a species-specific thermal dependence of running performance, should reduce niche overlap in sympatric forms (Brown and Wilson 1956). (2) The running performance of these two species should be either relatively insensitive to body temperature, that is lizards should perform relatively well over a broad range of body temperatures or (3) show a relatively narrow T_o at which running performance is maximized. In this last case, T_o should be the higher possible, according to the hypothesis of "warmer is better" (Angilletta et al. 2010). Therefore, lizards would select body temperatures that lead to sub-optimal running performance. This prediction is derived from the Jensen's inequality principle, that is based on the non-symmetrical physiological cost of thermal reaction norms. (Martin and Huey 2008). Instead, if support for (2) is found, then the distribution of T_{pref} should match running performance, indicating that lizards select body temperatures that maximize running performance.

Support of hypothesis (1) would indicate that the investigated species differ at the physiological level, possibly suggesting the action of a selective force to reduce niche overlap. Support for hypothesis (2) would indicate that these species are generalist regarding running performance and select body temperatures that allow maximum performance. Finally, if the hypothesis (3) is supported, there would be a mismatch between the optimum for running performance and the preferred temperature, potentially indicating a different evolutive pressure on these two physiological traits.

2. Methods

2.1 Organisms collection and housing

Lizards were collected during July 2016 by noosing near Moledo, a coastal locality in northern Portugal (41º50 N, 8º52 W) where the two species live in syntopy in a sandy area with rocks, sparse vegetation, and agricultural fields delimited by human-constructed stone walls. To avoid the effects of reproduction, body condition and ontogeny, only adult males with intact tails were captured (Carretero et al. 2005). For the locomotor experiment, we collected and measured the SVL (Snout-Vent length) of 20 adult males of each species. In the lab, lizards were housed inside individual terraria (40 cm×25 cm×25 cm) provided with a refuge, a source of water, and access to natural light. Food in the form of *Tenebrio molitor* larvae was provided every other day. To ensure that lizards' body condition did not change throughout the study, we measured the body mass of each lizard used to estimate performance curves both the day of capture and just before release. No mass loss during the experiment was detected (Paired t-test = 0.480, df = 39, P = 0.6341). The lizards used to estimate thermal preferences were collected in the same area and housed in the same way. After the experiment, all lizards were released at the site of capture.

2.2 Locomotor performance

We estimated locomotor performance as the maximum sprinting speed reached on a horizontal race track (1 m-long) at various body temperatures selected a priori to encompass lizards' activity range and include preferred temperature (Castilla et al. 1999). The substrate of the racetrack was made of cork, which provides excellent traction (Van Damme et al. 1997). We randomized the order of temperature and individual testing to avoid any potential effect of acclimation if trials were conducted, for example, at increasing temperatures. Hence, the order used was 33-25-28-36-38-20-31°C. Each day of testing, lizards were exposed to only one temperature and ran three times, resting at least two hours between trials to ensure physical recovery. This experimental protocol is typically used to ensure that maximal performance capacity is estimated (Losos et al. 2002). To assure that lizards run at the required body temperature, before each trial we placed them into a sealed incubator set at the desired temperature for one hour (Binder KB 53, precision at 25 and 40 °C: ± 0.2 and 0.3 °C, respectively). Additionally, we checked that lizards' body temperature matched the desired temperature before running (details in the next section). Each trial was recorded with a high-speed camera, set at 200 frames per second (Fastec IL3, resolution 1280 x 1024 pixels. Fastec Imaging San Diego, CA 92127 USA). The position of the lizard in each video frame was digitized using

MaxTRAQ 2D motion analysis software (Innovision Systems Inc. 2009). The highest instantaneous speed (Gomes et al. 2017) recorded across the three trials was taken as an estimate of each lizard's maximum performing capacity at that body temperature.

2.3 Pre-run body temperature

When we retrieved a lizard from the incubator and placed it on the race track, we quickly measured (within 10 s) its skin temperature by shooting a thermal picture using a FLIR T335 thermal camera (sensitivity: < 0.05°C; accuracy: ± 2% of the reading; IR image resolution: 320×240 pixels; FLIR Systems Inc., Wilsonville, Oregon, USA). Skin temperatures were afterward extracted from IR pictures using the Spotmeter function in FLIR Tools 5.12 (Copyright 2018 FLIR Systems, Inc; http://www.flir.com), selecting a spot in the last third of each lizards' back. That body position allowed us to avoid possible thermal heterogeneity in lizards' body and correlate well with internal (cloacal) body temperature (Sannolo et al. 2014, 2018). Such body temperatures were used for fitting performance curves.

2.4 Preferred body temperatures

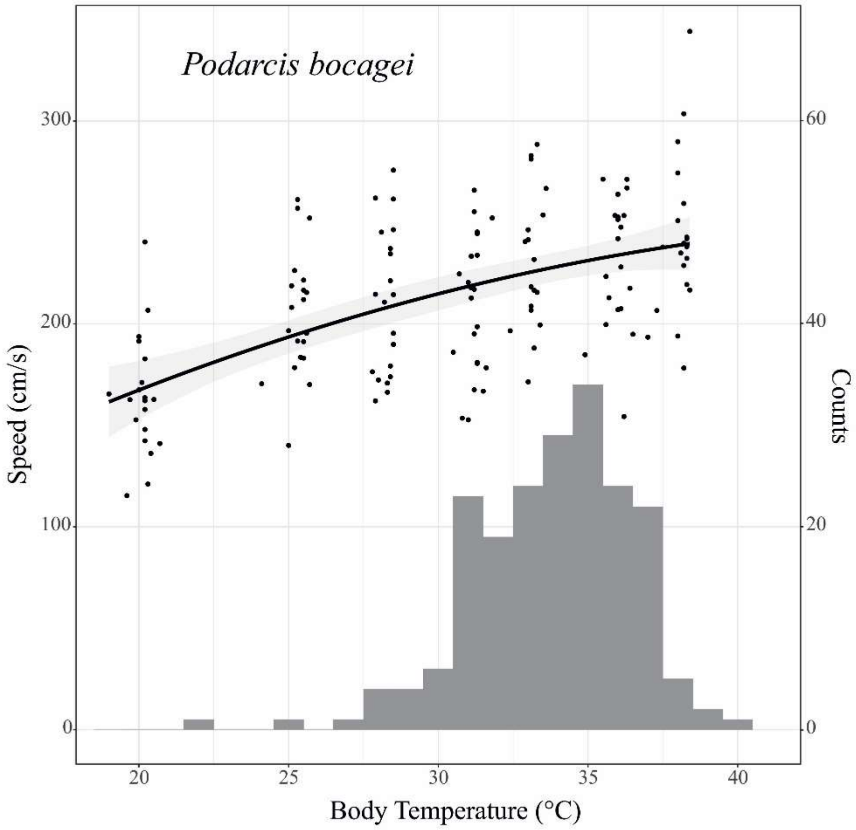
Data on the preferred body temperatures were obtained by randomly selecting 20 fully-hydrated adult male lizards of the same species used in a previous study on thermal preferences (Sannolo and Carretero, under revision). Merging data from different studies is common practice to compare performance curves and thermal preference. That is because it is challenging to avoid that one experiment does not bias the results of the second (Martin and Huey 2008). Briefly, we used linear thermal gradients following the method described in Barroso et al. (2016). Thermal gradients were set up in plastic containers (PVC; 100×30×40 cm) using sand as substrate (~1 cm deep). The gradients were kept in a room at 20°C and illuminated by natural light through a window. A 150 W infrared lamp suspended above one end of each gradient created a range of temperatures from approximately 20 to 50°C. Lizards were left undisturbed to explore the thermal gradient for one day. On the following day, we measured the skin temperature of each lizard every hour between 9.30 and 18.30 h (10 measurements) by shooting a thermal picture using the same thermal camera described earlier (a FLIR T335).

2.5 Data analysis

Before fitting and comparing performance curves, we first tested for the potential effects of individual variability. We compared the fit of a three-ways linear model (max performance ~ body temperature × species × SVL) against a mixed-effects models with the same variable structure, but which included individual identity as a random factor (Pinheiro and Bates 2000). Furthermore, because performance curves usually show a curvilinear trend, we compared the fit of the linear model against a quadratic model, using log-Likelihood ratio tests (LR). The potential interspecific difference in thermal preferences was assessed using a mixed-effects model (preferred temperature ~ species × SVL), again testing for the potential effect of individual variability. The same model was also run with body mass instead of SVL. To check if running performance reached a plateau and stopped increasing, we categorized the body temperatures into seven intervals (20-25-28-31-33-36-38-°C) and compared the maximum performance of each species using pairwise t-tests adjusted with Holm correction for multiple comparisons. To compare performance curves with the preferred temperatures, we contrasted the confidence intervals (95% CI) of the body temperatures selected in the gradients against the maximum sprinting speed. If CI did not overlap with maximum performance, we concluded that lizards did not match the two parameters. For the thermal preferences, we also calculated measures of the central tendency (mean and median), as well as measures of curve asymmetry (skewness). Mixed-effects models were fitted using the nlme package (Pinheiro et al. 2017). Statistical difference between models was assessed with the function anova.lme. Figures were produced with the ggplot2 package (Wickham, 2009). All statistical analysis was carried on in the R environment (R Core Team 2018).

3. Results

Individual variability played a significant role both in running performance (LR = 137.3, P < 0.0001) as well as in thermal preferences (LR = 26.13, P < 0.0001), as suggested by the random effects. Running performance was best represented by a quadratic trend rather than by a linear one (LR = 6.558, P = 0.0104), suggesting a steeper decrease in performance at lower body temperatures and an asymptotical increase at higher body temperatures (Fig. 5.2.1). The two investigated species showed similar performance curves, without differing at any specific body temperature (t-value = -1.757, P = 0.0874). The two species differed in their SVL (and body mass), with *P. bocagei* being longer than (two-tailed T-test = 4.653, df = 37, P < 0.0001) and heavier (two-tailed T-test = 6.602, df = 37, P < 0.0001) than *P. g. lusitanicus*. The fit of the model with either body mass or SVL performed similarly (AIC with SVL: 2627.3; AIC with body mass: 2629.2), and the lack of difference in running performance remained after either SVL or body mass as covariates were considered (SVL t-value = 1.809, P = 0.0787; body mass t-value = 1.369, P = 0.1793).



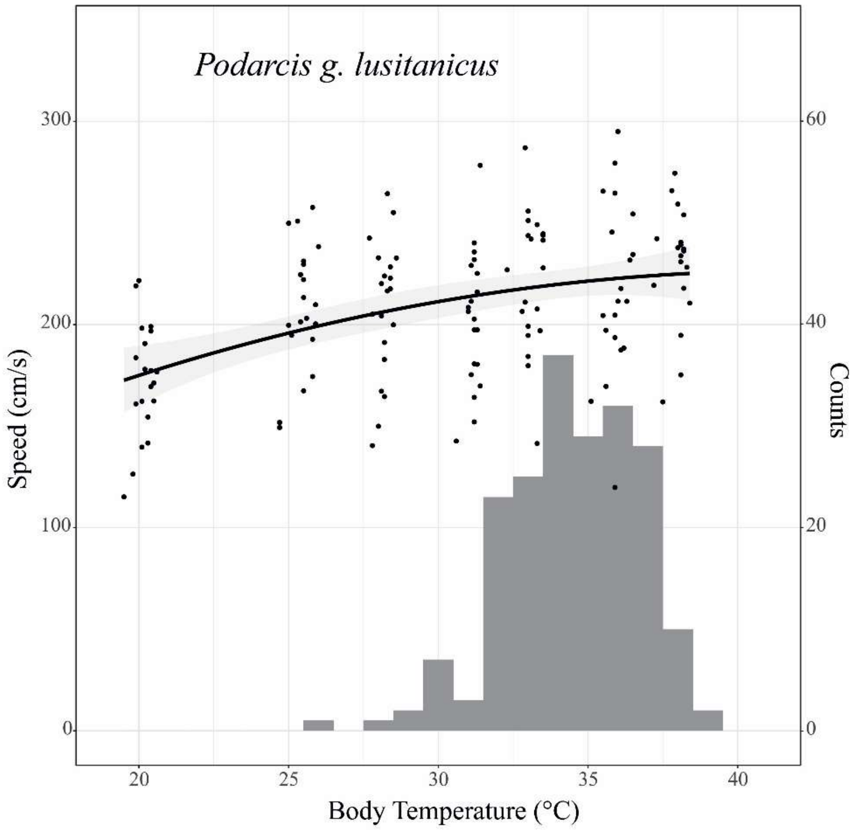


Fig. 5.2.1. Maximum running performance and thermal preference of *P. bocagei* and *P. g lusitanicus*. The left y-axis represents running performance expressed in cm/s (on a 1 m long race track). The right y-axis represents the counts for the histogram of preferred temperatures.

P. bocagei						
	20°C	25°C	28°C	31°C	33°C	36°C
25°C	**0.00424**	-	-	-	-	-
28°C	**0.00058**	1	-	-	-	-
31°C	**0.00154**	1	1	-	-	-
33°C	**<0.0001**	0.24978	0.58083	0.41416	-	-
36°C	**<0.0001**	0.21745	0.55060	0.37467	1	-
38°C	**<0.0001**	**0.01209**	0.06188	**0.02929**	1	1

Table 5.2.1. Pairwise comparisons of running performance of *P. bocagei* at various body temperatures. Holm correction for multiple testing was used. In bold are statistically significant values.

P. g. lusitanicus						
	20°C	25°C	28°C	31°C	33°C	36°C
25°C	**0.01566**	-	-	-	-	-
28°C	**0.01566**	1	-	-	-	-
31°C	0.08080	1	1	-	-	-
33°C	**0.00013**	1	1	0.79595	-	-
36°C	**0.00078**	1	1	0.37467	1	-
38°C	**<0.0001**	0.57956	0.57956	0.14972	1	1

Table 5.2.2. Pairwise comparisons of running performance of *P. g. lusitanicus* at various body temperatures. Holm correction for multiple testing was used. In bold are statistically significant values.

Within species, SVL played a marginal role in running performance for *P. bocagei* (t-value = -1.874, P = 0.069), while increasingly heavier lizards ran more slowly (t-value = -2.212, P = 0.033). For *P. g. lusitanicus*, instead, both SVL (t-value = 0.599, P = 0.5528) and body mass (t-value = -0.065, P = 0.947) had a non-significant effect on maximum performance. Such a result was likely the consequence of the small variation in SVL (all adult males, *P. bocagei* mean = 57.39 ± 3.13 mm; *P. g. lusitanicus* = 52.40 ± 3.64 mm) and body mass (*P. bocagei* mean = 4.19 ± 0.64 g; *P. g. lusitanicus* = 2.95 ± 0.54 g) of both species. Even though running performance was affected by body temperature in both species (*P. bocagei* t-value = 3.805, P = 0.0002; *P. g. lusitanicus* t-value = 3.804, P = 0.0002), lizards sprinted equally fast over a broad range of body temperatures. In *P. bocagei* the maximum performance reached a statistical plateau at 33°C (Fig. 5.2.1). Indeed, over this body temperature, the increase in running performance was not statistically significant (Table 5.2.1). In *P. g. lusitanicus* the statistical plateau was already reached already at 25°C (Table 5.2.2).

The preferred body temperature of the investigated species differed only slightly (t-value = 1.991, P = 0.0571), as already previously reported (Sannolo et al., 2018). The CI of the thermal preferences indicated that both species preferred temperatures higher than the plateau of running performance, but lower than the maximum tested temperature for running (*P. bocagei* mean Tpref = 33.8°C. CI = 33.5 – 34.2°C; *P. g. lusitanicus* mean Tpref = 34.5°C. CI = 34.2 - 34.8°C;). For both species, thermal preferences are negatively skewed (*P. bocagei* = -0.78; *P. g. lusitanicus* = -0.54). Body size apparently did not affect thermal preference in (*P. bocagei* t-value = 0.540, P = 0.593; *P. g. lusitanicus* t-value = -1.337, P = 0.1897), despite previous evidence that larger individuals selects higher body temperatures (Sannolo et al. 2018). As for performance, it should be remarked the reduced difference in SVL between the two species in the present statistical (*P. bocagei* mean SVL = 59.2 ± 3.49 mm; *P. g. lusitanicus* SVL = 56.1 ± 3.00 mm).

4. Discussion

We investigated here for the first time if two closely related lacertid lizards, which largely overlap in their distribution range, differ in their running performance at various body temperature. We also assessed if thermal preferences matched the optimum for maximum running performance. We found little difference in running performance of the two species. Such a negative result may suggest a lack of intrinsic variation for this trait to evolve, not enough time for evolutionary divergence or a strong stabilizing selective pressure on this trait. Of the two alternative hypotheses regarding the broadness of running performance, we found support for the insensitivity of running performance to body temperature. The results indicated that both species performed well over a broad range of body temperatures. Finally, thermal preferences largely overlap with the temperature at which running performance is maximized.

Our first result indicated that these two species are characterized by very similar running performance. We did not detect interspecific differences although the two species differ in morphology (Kaliontzopoulou et al. 2012), habitat selection, and in some locomotory tasks (Gomes et al. 2016). Similarly, despite small differences, thermal preferences were similar, as already reported in the literature (Sannolo et al. 2018). Depending on the species and the evolutionary context, studies have found support for both inter- and intraspecific variation of thermal sensitivity of performance curves as well as rigidity in this trait (Hertz et al. 1983; Crowley, 1985; Huey and Kingsolver 1989). Indeed, depending on the considered function, thermal physiology is sometimes viewed as an evolutionarily rigid or labile trait (Hertz et al. 1983). When thermal sensitivity of performance differs among populations or species, such a divergence is usually attributed to adaptation, because the available data suggest that the capacity for acclimation is limited (Bennett 1990). The present case study supports a conservative view on the thermal performance in sympatric lacertid lizards. However, the thermal sensitivity of other locomotor performances might diverge (Angilletta et al. 2002). For example, at a constant temperature, the species tested here diverged in their climbing performance and maneuverability (Gomes et al. 2016). Similarly, although we found support for a significant intraspecific variation in running performance in both species, such an effect is not attributable to the small variability in body size. Our data did not allow us to examine more in-depth potential patterns of individual variation, that would require a much larger data set (Mitchell et al. 2016). Further studies on this subject should focus with more emphasis on the individual contribution, that eventually may shape the specific form of thermal reaction norms.

The second result of this study indicates that running performance of both species is relatively insensitive to changes in body temperature, at least within the temperature ranges at which lizards are expected to be active in the field. Such a result coincides with the previous finding for other lizard species (Angilletta et al. 2002; Bennett 1980; Huey et al. 1989). Physiological functions that are rather insensitive to temperature, like running performance, are better described by optimal temperature ranges (Huey and Stevenson 1979; Angilletta et al. 2002). In the present case, *P. bocagei* sprints maximally in a temperature range comprised between 33-38°C, while *P. g. lusitanicus* sprints maximally in the range 25-38°C. Hence, even though we did not find statistical support for interspecific differences in the slope of the running curve, it appears that *P. g. lusitanicus* has a shallower running curve than *P. bocagei*, and can perform nearly at maximum over a range of 13°C. Running performance of lacertid lizards might be more sensitive to body temperature at extremes body temperature, both low and high, as already suggested for phrynosomatid lizards (Angilletta et al. 2002). Indeed, our data support a decrease in running performance below approximately 25°C of body temperature. Similarly, it is likely that performance over 38°C might start to decrease. Even though no data is available on the minimum and maximum body temperature at which these species are active in the field (*P. bocagei* 22 – 36 °C, Carretero unpublished data; *P. g. lusitanicus* 15 – 40 °C Pérez-Mellado 1983), we suspect that such sensitivity likely coincides with the lower and upper limit of lizards' activity. Thermal preferences in the lab support this speculation, because *P. bocagei* selected body temperatures below 26.8°C only in two occasions, and *P. g. lusitanicus* never selected values below 26.5°C (Fig. 5.2.1). Hence, when lizards can choose body temperature without constraints, they avoid values that lay outside the range of maximum sprint speed. The available literature suggests that typically lizards do not use the full potential of their locomotor capacity in daily life (Hertz et al. 1988).

Even though running performance seemed relatively insensitive to temperature and thermal preferences appear to be a conservative trait in these two species, we have currently no data on the potential variability across populations neither on thermal preferences nor on locomotor performance. In other lizard species, inter-populational variability, as well as conservativeness, has also been found on both thermal preferences (Yang et al. 2008; Zamora-Camacho et al. 2016) and performance (Crowley 1985; Angilletta et al. 2002; Zamora-Camacho et al. 2015). Hence, further studies on thermal biology of Podarcis species should focus on the potential coadaptation of thermal preferences and performance, especially along environmental gradients (Van Damme et al. 1989).

Concluding, the running performances of the two species tested here are very similar, suggesting little or no divergence in this whole-organism trait. We argue that, despite the ecological and morphological differences reported for these two species, strong selective pressures related with the daily need for optimal locomotor performance to move, feed, escape from predators and reproduce, likely prevented any significant divergence between these two species. Finally, performance curves are insensitive to body temperature in these species, and preferred temperatures selected in the gradients allow lizards to run at their maximum. Despite little variation in body size, individual variability accounted for a significant portion of variation, and more investigation should be devoted to that aspect. Similarly, little information is available on the relationship between body temperature and performance in the field. For example, to what extent lizards run at their maximum in the field is still poorly investigated (Braña 2003).

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Chapter 6: The effects of dehydration on thermal preferences and variability in resistance to water loss

"Biology has forgotten water, or never discovered it"

— Albert Szent-Gyöorgyi (1971)

6.1 Article V: Dehydration constrains thermoregulation and space use in lizards

PlosOne - *In revision*.

Marco Sannolo, Miguel A. Carretero

Abstract

Climate change is negatively affecting many species. The increase in mean air temperature is often associated with shifts in distribution, changes in phenology, and local extinctions. Other factors that only partially correlate with air temperature, like water and food shortage, may also contribute to the negative consequences of climate change. Although the effect of temperature on lizards' ecophysiology is highly studied, many lizards are also at risks of increased water loss and dehydration, which are predicted to increase under climate change. Here we aimed for the first time to explore if lacertid lizards exposed to acute dehydration thermoregulate less precisely than hydrated lizards and if dehydrated lizards are less active, change the daily pattern of thermoregulation and balance water balance against thermoregulation. We exposed four lizard species with differences in the thermal preference to thermal gradients with or without a source of water. We measured preferred body temperatures, daily pattern of thermoregulation, and the use of space. Dehydration negatively affected thermoregulation in all investigated species. Dehydrated lizards reduced their preferred body temperature and showed a species-specific pattern of hourly change in thermal preference. Furthermore, they more frequently used the colder parts of the gradients and spent more time hidden. Lizards experiencing acute dehydration may suffer a reduction in survival and fitness because of poor thermoregulation. Similarly, they may spend more time hidden, waiting for more favourable weather conditions. Such inactivity may carry ecological costs especially in those regions that undergo either short or prolonged periods of droughts.

1. Introduction

Changing climatic conditions over the past decades are affecting many animal species (Parmesan 2006). As a result, many taxa are experiencing range shifts (Moritz et al. 2008), change in phenology (Parmesan and Yohe 2003), and local extinction (Wiens 2016) among other effects. However, climate change rate is not globally uniform (Urban 2015), and some regions are experiencing a faster rise in temperature than others (Loarie et al. 2009). Furthermore, extreme climatic events, like heatwaves (Schär et al. 2004) and droughts (Dai 2013) are becoming more common in some regions, like the Mediterranean basin (Rahmstorf and Coumou 2011). Similarly, some species are more exposed to climate change either because they live in areas from which they cannot escape, like mountaintops (Huey et al. 2009), or because they are particularly vulnerable to the effect of rising temperatures and extreme climatic events, like many amphibians (Kiesecker et al. 2001).

Reptiles depend strongly on the local external environment to maintain their body temperature within a well-defined range in which performance is optimal (Huey 1982; Angilletta 2009). Lizards are receiving much attention in recent years since they are considered particularly vulnerable to abrupt changes in environmental temperature (Huey et al. 2010) that might reduce their activity budget (Deutsch et al. 2008) and lead to local extinctions (Sinervo et al. 2010). Indeed, lizards appear to be particularly at risk, as some estimate that up to 20% of lizard species may be at risk of extinction by 2080 (Sinervo et al. 2010; but see Huey et al. (2012) for the role of behaviour and habitat in modulating the effect of climate change in ectotherms). The increase of mean air temperature is often used as the primary argument for explaining the adverse effect of climate change on animal populations, including lizards (Sinervo et al. 2010; Rosenzweig et al. 2008). However, there is only partial support for the increase in air temperature as being the primary cause of species extinction (Cahill et al. 2012). Other factors, which partially covary with air temperatures, like food and water availability, are likely among the primary variables determining the survival of a given population under climate change (McLaughlin et al. 2002).

While the thermal ecology and physiology of lizards have been extensively studied for almost a century (Cowles and Bogert 1944; Angilletta et al. 2002a; Angilletta 2009), much less is known on the negative consequences of water shortage on lizard behaviour, ecology, and conservation. At the individual level, for example, a recent experiment conducted in a confined environment showed that the rainfall regime affected lizards' behaviour and activity. In particular, during dry periods lizards used shaded microhabitats more often, while they were more active in sunny microhabitats following rainfall (Ryan et al. 2016). In general, it seems that dehydrated lizards select for lower body temperatures, probably to contain water loss (Crowley 1987). In temperate regions, water shortage or droughts may also affect lizards' reproduction (Wang et al. 2016) and development, with yearlings exposed to dry condition showing lower growth and activity rates (Lorenzon et al. 1999). At the population level, lizards might be able to physiologically adjust their resistance to water loss in function of the availability of free-standing water (Dupoué et al. 2017).

To complicate the matter, a warmer and drier environment may affect lizards in complicated ways since the interactions between thermal and water balance, and how lizards react to optimize them are not clear. For example, a thermoregulating lizard may get dehydrated, which leads to an excessive concentration of plasma solutes (Minnich 1982). On the other hand, while keeping suboptimal body temperature may prevent dehydration, it also leads to reduced locomotor and foraging performances (Avery and Mynott 1990; Angilletta et al. 2002b). Furthermore, geographically separated populations or closely related species might react differently to dehydration, reflecting local adaptation or plasticity (Dupoué et al. 017). Climate change may further blur the picture because some species or populations might be exposed to different patterns of increasing temperature and decreasing water availability. To what extent dehydrated lacertid lizards prioritise either thermoregulation or reducing water loss is still unknown and deserves further investigation.

Here, we aimed to assess the impact of acute dehydration on lizard thermoregulation. In particular, we quantified the effect of dehydration on lizard thermoregulation using four Podarcis species as a case study (*P. bocagei*, *P. carbonelli, P. guadarramae*, and *P. virescens*). These lacertid species form a single clade that originated in the western Iberian Peninsula during the late Miocene (Kaliontzopoulou et al. 2011), in an epoch during which the climate was more humid, cooler, and summer droughts were yet to come (Jiménez-Moreno et al. 2010). These species have been recently studied regarding thermal ecology (Barroso et al. 2016) and resistance to water loss (Sannolo et al. 2018). For instance, sympatric and sister taxa species differ in both thermal preference and resistance to water loss (Barroso et al. 2016). Further, these species show notable differences regarding habitat use (Gomes et al. 2016) and morphology (Kaliontzopoulou et al. 2012). They also differ in their distribution patterns, with *P. bocagei* and *P. carbonelli* restricted to areas with Atlantic influence, while *P. guadarramae* and *P. virescens* tend to occupy more Mediterranean environments, although they may partially overlap either regionally or locally (Loureiro et al. 2008). Their context of evolutionary history and ecology make *Podarcis* wall lizards an excellent system to investigate not only the impact of dehydration on thermoregulation, but also its variation across a clade of closely related, yet very distinctive species.

We formulate three testable hypotheses on the consequences of dehydration on thermal ecology of lizards. We expect that (1) dehydrated lizards will reduce their body temperature, to prevent or diminish further water loss, mainly by using more frequently cooler areas or spending more time hiding; that (2) thermoregulation will be more precise (the mean deviation from preferred temperature is smaller) when lizards have access to water (because lizards can optimize thermoregulation); and that (3) dehydrated lizards are forced to physiologically trade-off body temperature and water retention. When water is not available, lizards keeping higher body temperature should lose proportionally more water than those that reduce their body temperature to maximize water retention.

2. Methods

2.1. Sampling and housing

Lizards were collected from two localities in Northern Portugal. In each, a pair of species can be found living in syntopy. We sampled *P. bocagei* and *P. guadarramae* near Moledo, a coastal region in Viana do Castelo province (41º50’ N, 8º52’ W), while we sampled *P. carbonelli* and *P. virescens* in Santa Maria da Feira, in Aveiro province (40º55’ N, 8º32’ W). Sampling spanned most of the activity season for these species in those locations (beginning of April – end of September). We captured only adult males with intact tails, to avoid reproductive status, body condition and ontogeny affecting thermal preferences (Carretero et al. 2005). We identified each individual by means of photo-identification, to avoid pseudoreplication (Sacchi et al. 2016). Before the experiment, we measured the snout-vent length (SVL) of each lizard to the nearest 0.01 mm with a digital calliper. We then left lizards undisturbed for 24 h in individual plastic cages (40×25×25 cm) and provided them with a source of water but with no food. After the first 24 h, we moved each lizard to a randomly chosen gradient (see below) for acclimation. We assumed that when the experiment started, lizards were fully hydrated, having had access to unlimited water during the previous 24 h.

2.2. Preferred body temperature

We assessed the preferred body temperatures of lizards in linear thermal gradients following the method of (Barroso et al. 2016). Thermal gradients were set up in plastic containers (PVC; 100×30×40 cm; Fig. 6.1.1) using sand as substrate (~1 cm deep). The gradients were kept in a room at 20 °C and illuminated by natural light through a window. A 150 W infrared lamp suspended above one end of each gradient created a range of temperatures from approximately 19 to 68 °C (Table S8). At the cooler end of each gradient, a small plastic box (13×8.5×5.5 cm) provided a refuge. We placed each lizard in a thermal gradient at 19.00 h when lights were off. The following morning, the infrared lights turned on at 08.00 h and turned off again at 19.00 h. On the first day, lizards were left undisturbed to explore the thermal gradient. At the end of the first day, we randomly emptied the water source (a plastic lid, ~13 ml) from half of the gradients and left it in the other half. On the next (second) day, the lamps were turned on during the same time span. Every hour between 9.30 and 18.30 h (10 measurements) we measured the skin temperature of each lizard by shooting a thermal picture using a FLIR T335 thermal camera (sensitivity: < 0.05°C; accuracy: ± 2% of the reading; IR image resolution: 320×240 pixels; FLIR Systems Inc., Wilsonville, Oregon, USA). For consistency, the same person (MS) shot all the photos. Skin temperatures were extracted from each IR picture using the Spotmeter function in FLIR Tools 5.12 (Copyright 2018 FLIR Systems, Inc; http://www.flir.com), selecting a spot in the last third of each lizards' back. This position allowed us to avoid thermal heterogeneity in lizards' body and correlate well with internal (cloacal) body temperature (Barroso et al. 2016; Sannolo et al. 2014). At the end of the second day we reversed the treatments (the water was added to those gradients without it and removed from those gradients where it was present) and measured the hourly skin temperatures of each lizard during the following morning as described above.

2.3. Spatial use of the gradients

Along with their main axis, gradients were divided into five sections of 20 cm each, visually identified by narrow strips of tape running along the walls (Fig. 6.1.1). By dividing each gradient in this manner, we created five virtual sections (plus the refuge) characterized by different available temperatures, to assess whether lizards used the various areas differently depending on the treatment. Upon shooting each IR picture, we noted the position of the lizard inside the gradient, with respect to the five sections. If a lizard was inside the refuge, we noted this as a sixth position. By using such system, we were able to reference the spatial (linear) use of the gradients with a resolution of 20 cm and with one body temperature measurement every hour for each lizard.

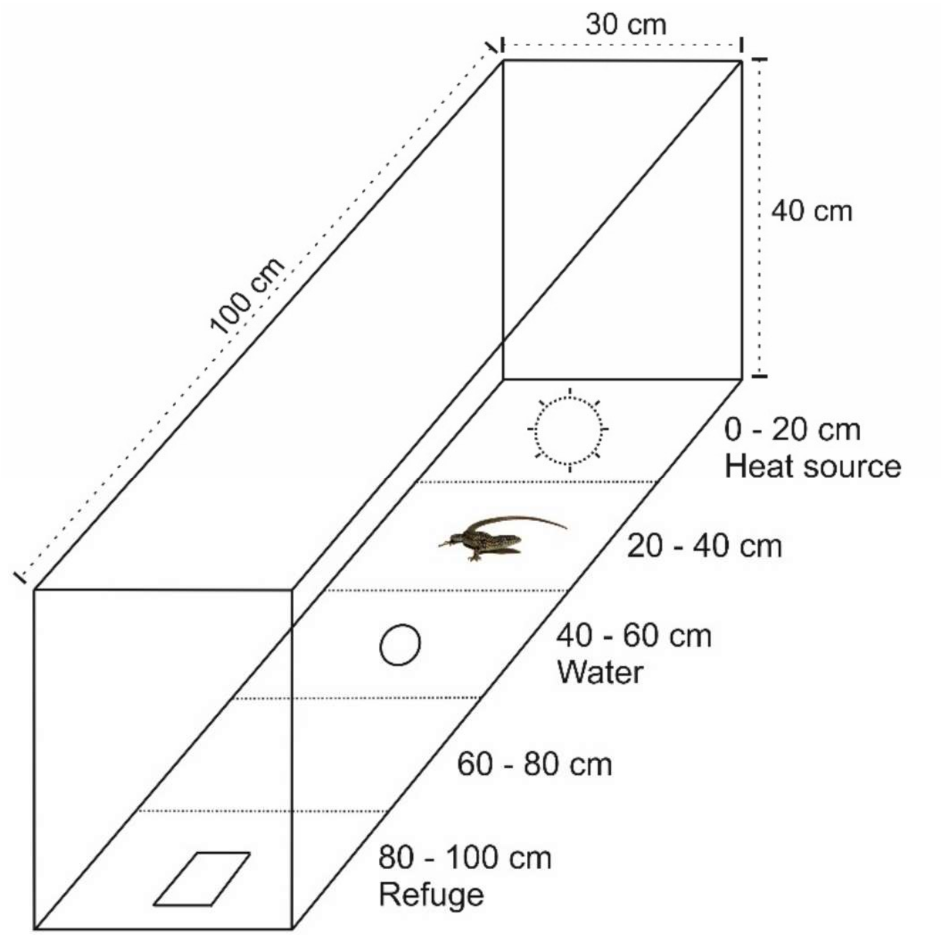

Fig. 6.1.1. Scheme of the experimental setting used in this study. The five sections, 20 cm each, were only virtual and lizards had full access to every spot in the gradient. Just as an example, a lizard is depicted during thermoregulation in the most used section (see results). The two treatments differed only by the water container being full or empty.

2.4. Body mass measurements

At the end of the second and third day (after taking the last thermal picture, at ~19.00 h), each lizard was briefly removed from its gradient and weighted to the nearest 0.1 mg using a precision balance (Sartorius M-Pact AX224, Sartorius AG, Goettingen, Germany). After the second day, lizards were released back into the gradients, while after the third day the experiment ended, and we left them for 24 h in individual boxes with water provided ad libitum. Subsequently, all lizards were released back to their respective site of capture. The difference in body weight between the control and experimental day (with or without access to water) was used to calculate the percentage of water loss (divided by the mass of the assumed fully hydrated lizard) for each individual. Since lizards did not have access to food during the entire period of the experiment, we ruled out that faeces (that are usually expelled in association with urine) might contribute to water loss. We confirmed such assumption by inspecting the gradients after the experiment. We were not able to find any residue that might suggest defecation.

2.5. Statistical analysis

To quantify the effect of dehydration, species and time of the day on thermal preferences, we fitted a mixed-effects model with body temperature as the dependent variable and the triple interaction treatment × species × time as the predictor. Because body temperature may vary non-linearly with time depending on the treatment, the variable "time" was fitted both as linear and quadratic terms, and the statistical difference of the resulting models was tested using log-likelihood ratio tests (LR) with the function anova.lme. The model with time as a quadratic term fitted the data better and hence was used for subsequent analysis (LR = 178.4, $P < 0.0001$) To account for repeated measures of the same individual and the possible temporal autocorrelation of the measurements, we included and tested for the effect of individual identity as random factor and repeated measures nested within individuals as autocorrelative structure. The difference between the models with and without random effect was significant (LR = 123.8, $P < 0.0001$) and, hence, random effects were retained. Instead, models with or without autocorrelation structure were not different (LR = 0.833, P = 0.36). Hence, repeated measures were considered as statistically independent in subsequent analysis. The contribution of main factors and their interaction was assessed inspecting t-tables from models' summary. We also fitted a linear model to test for a possible negative interaction between the amount of water loss and the resulting thermal preference (model form: percentage of water loss ~ mean body temperature (when dehydrated) × species, no random effect, each individual used only once). Percentages of water loss accounted for interindividual differences in body size and were normalised using square root (Shapiro-Wilk normality test (W) = 0.99, P = 0.55). We tested the significance of skewness in body temperature using the D'Agostino test. We tested for the decrease in thermoregulation precision by comparing the variances of body temperatures using F-tests. For both D'Agostino and F-tests, we accounted for repeated measures using the function p.adjust, set on 10 replicates and using the correction of Benjamini and Yekutieli (2001). Skewness values, both original and corrected, are reported in Table S6. We tested if the use of the space was different under the two treatments using Chi-square tests with the R built-in function chisq.test. To test for interspecific differences in SVL and body mass, we used ANOVA. Statistical analysis was run in R version 3.5.0 (R Core Team 2017). We fit mixed-effects models with the nlme library (Pinheiro et al. 2017), D'Agostino tests with the moments library (Komsta and Novomestky 2015), Cohen's d effect sizes with the effsize library (Torchiano 2017) and figures were produced using the library ggplot2 (Wickham 2009). Reported values represent mean ± standard deviation (SD) unless otherwise specified.

3. Results

2.1. Preferred body temperatures

We tested 116 individuals, collecting a total of 2320 body temperature measurements (Table 6.1.1). When lizards did not have access to water, all species reduced significantly (*P. bocagei* t-value = 9.114, d.f. = 550, P < 0.0001; *P. carbonelli* t-value = 7.101, d.f. = 474, P < 0.0001; *P. guadarramae* t-value = 10.35, d.f. = 596, P < 0.0001; *P. virescens* t-value = 8.859, d.f. = 607, P < 0.0001; Fig. 6.1.2) their mean body temperature, as well as the median and the mode of the distribution. Changes in curves distribution were more marked if mean values are considered (Table 6.1.1). The effect sizes (Cohen 1988) ranged from moderate to large (Table 6.1.1), suggesting that one day without access is enough for the onset of dehydration. Even though some species differed from each other in their preferred body temperature when water was available (Table S2), such differences turned not significant when water was not available. As predicted, all species thermoregulated more precisely when fully hydrated (*P. bocagei* F-test = 0.292, d.f. =289, P <0.0001; *P. carbonelli* F-test = 0.289, d.f. =249, P <0.0001; *P. guadarramae* F-test = 0.186, d.f. =299, P <0.0001; *P. virescens* F-test = 0.264, d.f. =319, P <0.0001; Table 6.1.1). Furthermore, the effect of dehydration seemed mostly related to the statistical distribution of the data, rather than a shift in the preferred (mean) temperature (note the overlap of the open, dashed arrows in Fig. 6.1.2). Indeed, body temperatures were left-skewed for all species and in both treatments (P < 0.01 in all cases, Table S1). However, the skewness of the dehydrated lizards' body temperature distribution curves increased, while peaks of distribution changed to a lesser extent (Table 6.1.1).

2.2. Hourly pattern of body temperatures

When the daily effect of dehydration on thermoregulation was considered, that is the interaction treatment × time, and we found that its effect was significant for all species (*P. bocagei* t-value = 3.308, d.f. = 546, P = 0.001; *P. carbonelli* t-value = 2.769, d.f. = 470, P = 0.006; *P. guadarramae* t-value = 4.355, d.f. = 565, P < 0.0001; *P. virescens* t-value = 3.724, d.f. = 603, P = 0.0002). Such a result indicates not only that mean body temperature was different between treatments for all species, but also that such a difference changed along the day (Fig. 6.1.3).

			Preferred body temperature (°C)						
Species	n	Treatment	Mean	Median	Interquartile range (75% - 25%)	Mean diff.	Median diff.	Mode diff.	Cohen's d (mean diff.)
P. bocagei	29	Water	33.8 ± 2.7	34.2	35.6 – 32.2	2.8	1.9	1.5	0.83
		No Water	31.0 ± 5.0	32.3	34.6 – 28.2				
P. carbonelli	25	Water	34.2 ± 2.3	34.3	35.8 – 32.9	2.2	1.25	0.1	0.60
		No Water	32.0 ± 4.3	33.1	35.1 – 30.1				
P. guadarramae	30	Water	34.6 ± 2.4	34.9	36.3 – 33.2	3.4	1.8	0.8	0.79
		No Water	31.2 ± 5.5	33.1	35.2 – 27.7				
P. virescens	32	Water	33.8 ± 2.5	33.8	35.6 – 32.5	2.6	1.3	-0.7	0.68
		No Water	31.2 ± 4.9	32.5	34.6 – 29.6				

Table 6.1.1. Comparison of mean, median and interquartile range of the preferred body temperature of four lacertid (*Podarcis*) species when water was either available or not in the gradients. Values after ± represent the standard deviation. Paired Cohen's d for the mean difference are calculated after Cohen (1988).

		Body mass (g)					
Species	SVL (mm)	With water	No water	Difference g (%)	*t*- value	*P*	Cohen's d
P. bocagei	58.61 ± 3.68	4.10 ± 0.80	3.75 ± 0.74	0.35 (8.5)	10.58	< 0.0001	1.97
P. carbonelli	53.19 ± 4.40	2.74 ± 0.71	2.43 ± 0.65	0.31 (11.3)	10.31	< 0.0001	2.06
P. guadarramae	56.09 ± 3.33	3.33 ± 0.56	3.08 ± 0.55	0.25 (7.6)	19.21	< 0.0001	3.51
P. virescens	56.84 ± 4.30	3.34 ± 0.76	3.09 ± 0.71	0.25 (7.5)	9.08	< 0.0001	1.60

Table 6.1.2. Body mass after 24 h either with or without access to water in the gradients. Differences in body mass are expressed both in grams and in percentages. Values are rounded to the second decimal value.

More precisely, when water was not available, all species adopted a strong curvilinear strategy, with higher body temperatures selected during the central part of the day and lower temperature at the beginning and the end of the day. Still, even during the central part of the day, when dehydrated lizards thermoregulated the most, the difference between treatments was still significant at every time interval, as suggested by the 95 % C.I. (Fig. 6.1.3).

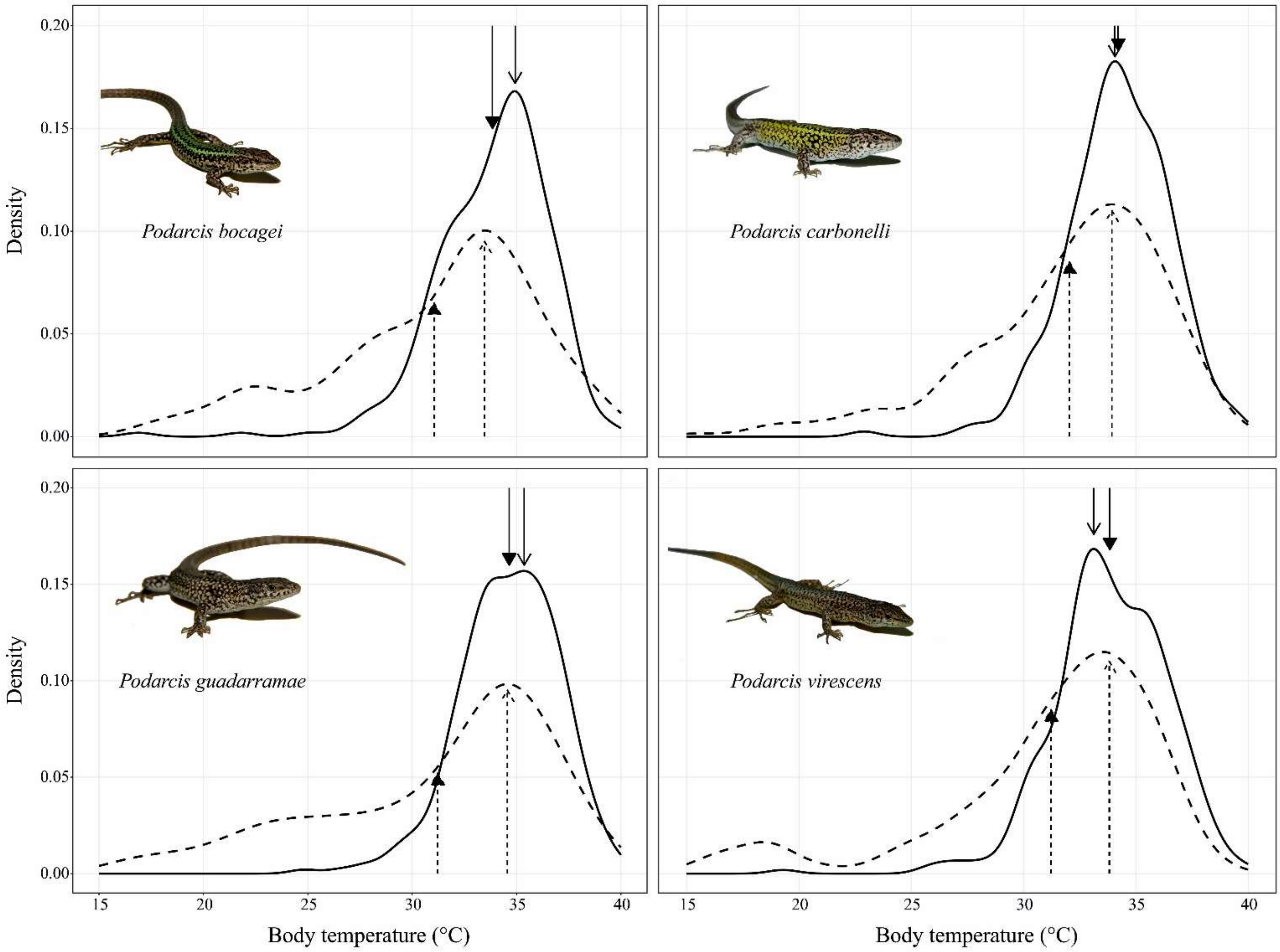

Fig. 6.1.2. Distributions of the body temperatures selected by four lacertid species when water was either available in the gradient (solid lines) or not (dashed lines). Y-axis represents standardized (density) frequency of use for each body temperature. Closed arrows represent mean values; open arrows represent the modes (see Table 6.1.1 for the numerical values.

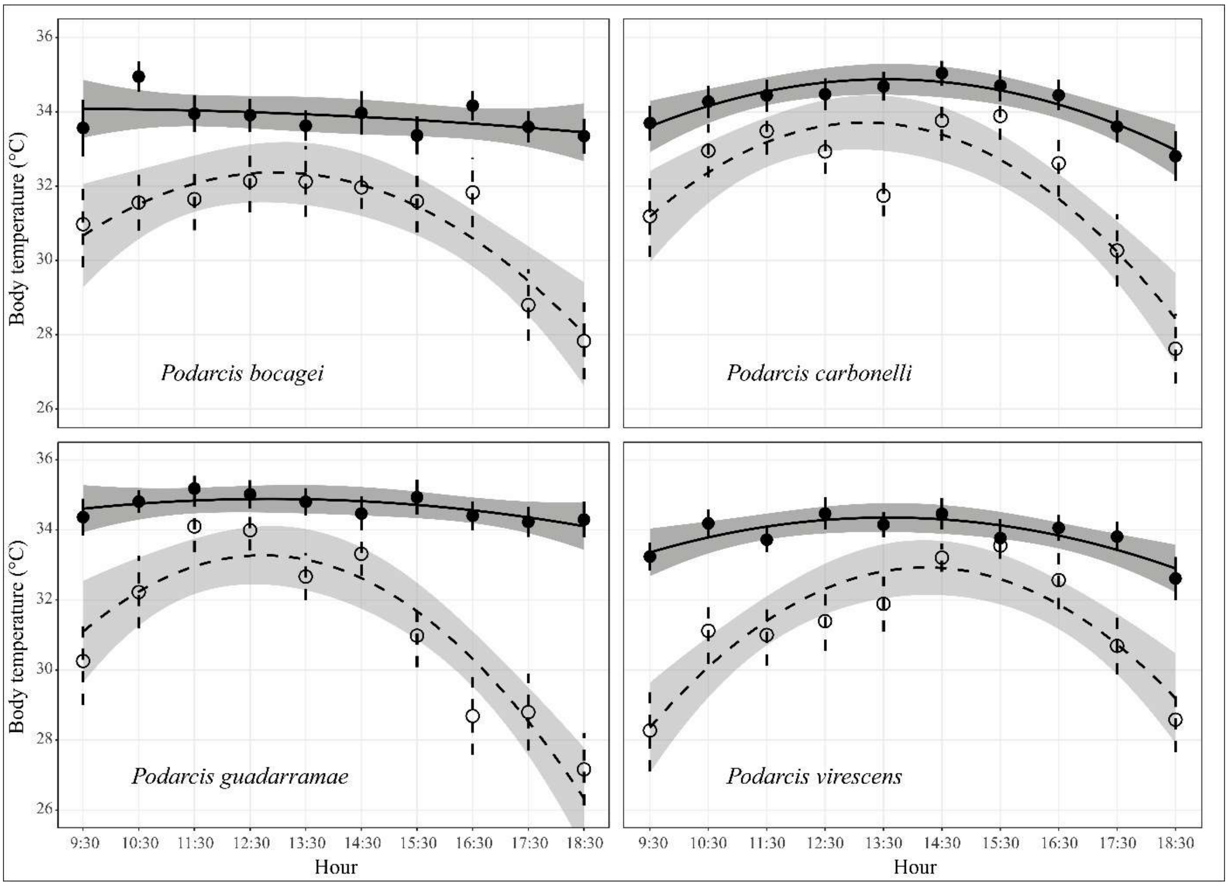

Fig. 6.1.3. Hourly-based body temperature selected by the four lacertid species when water was either available in the gradient (solid lines and dots) or not (dashed lines and triangles). Vertical bars represent standard errors. Shaded areas represent 95 % confidence intervals.

2.3. Spatial use of the gradients

All species exploited the gradients differently depending on the treatment (*P. bocagei*: $\chi 2$ = 83.03, d.f. = 5, P < 0.0001; *P. carbonelli*: $\chi 2$ = 40.16, d.f. = 5, P < 0.0001; *P. guadarramae*: $\chi 2$ = 98.4, d.f. = 5, P < 0.0001; *P. virescens*: $\chi 2$ = 48.24, d.f. = 5, P < 0.0001). Lizards under both treatments spent most of their time within 20-40 cm from the lamp (Fig. 6.1.1 and Fig. 6.1.4). Such position offered the best spot for thermoregulation, with an environmental temperature of ~30°C (Fig. 6.1.4 and Table S3). The hottest spot in the gradient, directly under the lamp, was used more by hydrated lizards. On the contrary, the refuge, situated in the cool part of the gradient, was mainly used by dehydrated lizards (Fig. 6.1.4). Lizards scarcely used intermediate sections, regardless of the treatment. Thus, they spent most of their time in the most favourable spot for thermoregulation, but when water was available, they also exploited the warmest area of the gradient since dehydration was not a limiting factor for thermoregulation. Conversely, lizards suffering from dehydration often sought refuge inside the shelter.

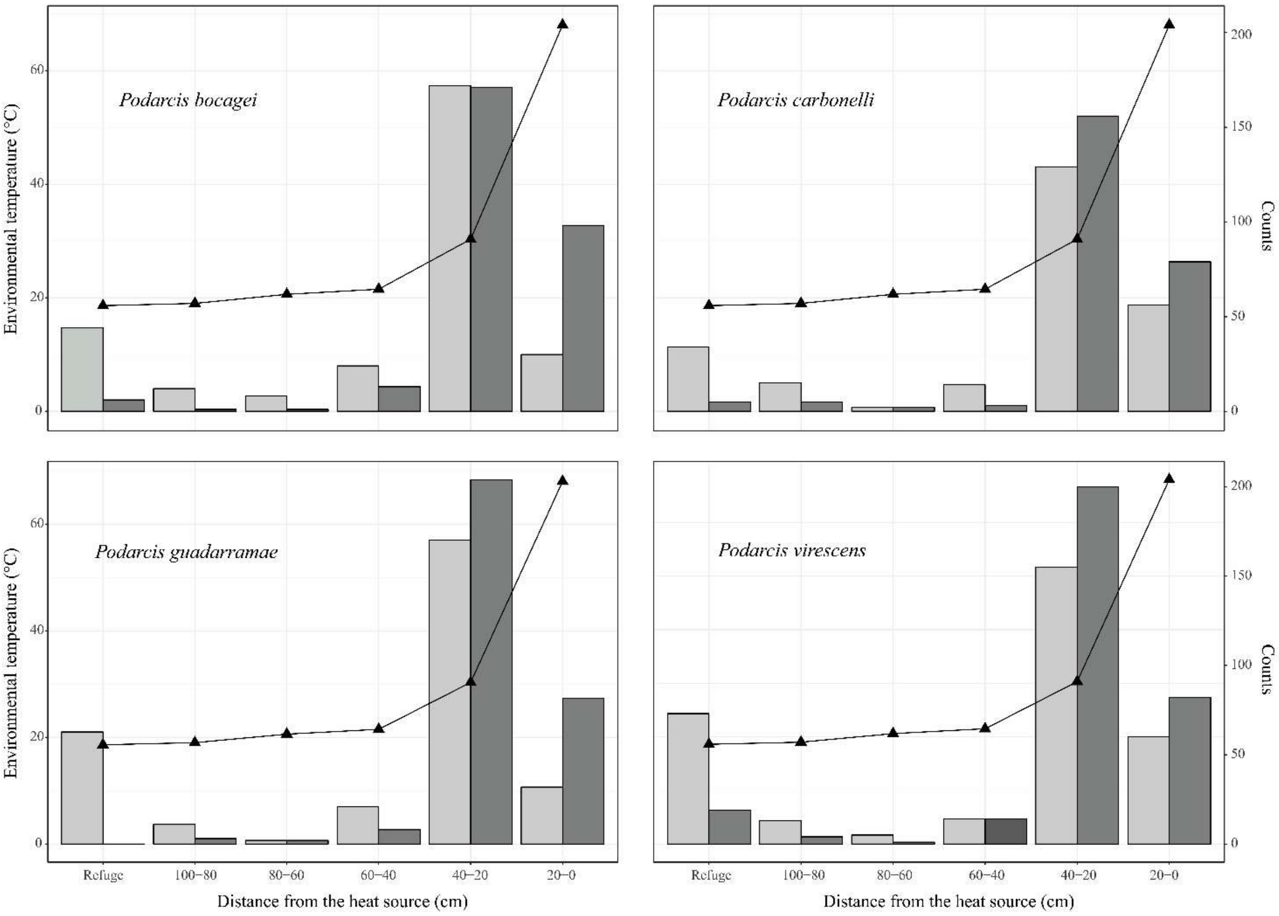

Fig. 6.1.4. Spatial use of the gradients of four species of lacertid lizards. The x-axis represents the six areas into which each gradient was virtually divided. The leftmost part represents the refuge location, while the rightmost position was directly below the heating lamp. The left y-axis represents the available temperature in the gradient for each position (Table S8, Supporting Information for details on how it was estimated). The right y-axis represents the count of lizards for each position. Dark grey bars were counts for lizard when water was available in the gradient, while light grey bars represent lizards that had no access to water.

2.4. The effect of dehydration on body mass

The four species of lizards differed in SVL ($F_{3,112} = 8.771$, $P < 0.0001$) and initial body mass ($F_{3,112} = 16.6$, $P < 0.0001$). After 24 h of thermoregulation without access to water, all species lost a significant amount of body mass (Table 6.1.2). At the end of the experiment, the most dehydrated species, relative to its body mass, was *P. carbonelli* (11.3 %), while the less dehydrated was *P. virescens* (7.5 %; Table 6.1.2). The effect size (Cohens' d) was large for all species, suggesting that 24 h of water shortage are enough for imposing dehydration. The loss of body mass did not solely depend on body size. Indeed, even though the smallest species (*P. carbonelli*) suffered the most from dehydration, the biggest species (*P. bocagei*) was not the better in coping with water loss. Such result is reinforced by investigating the species-specific relationship linking water loss (as a proportion of body mass) and preferred temperature (Fig. 6.1.5).

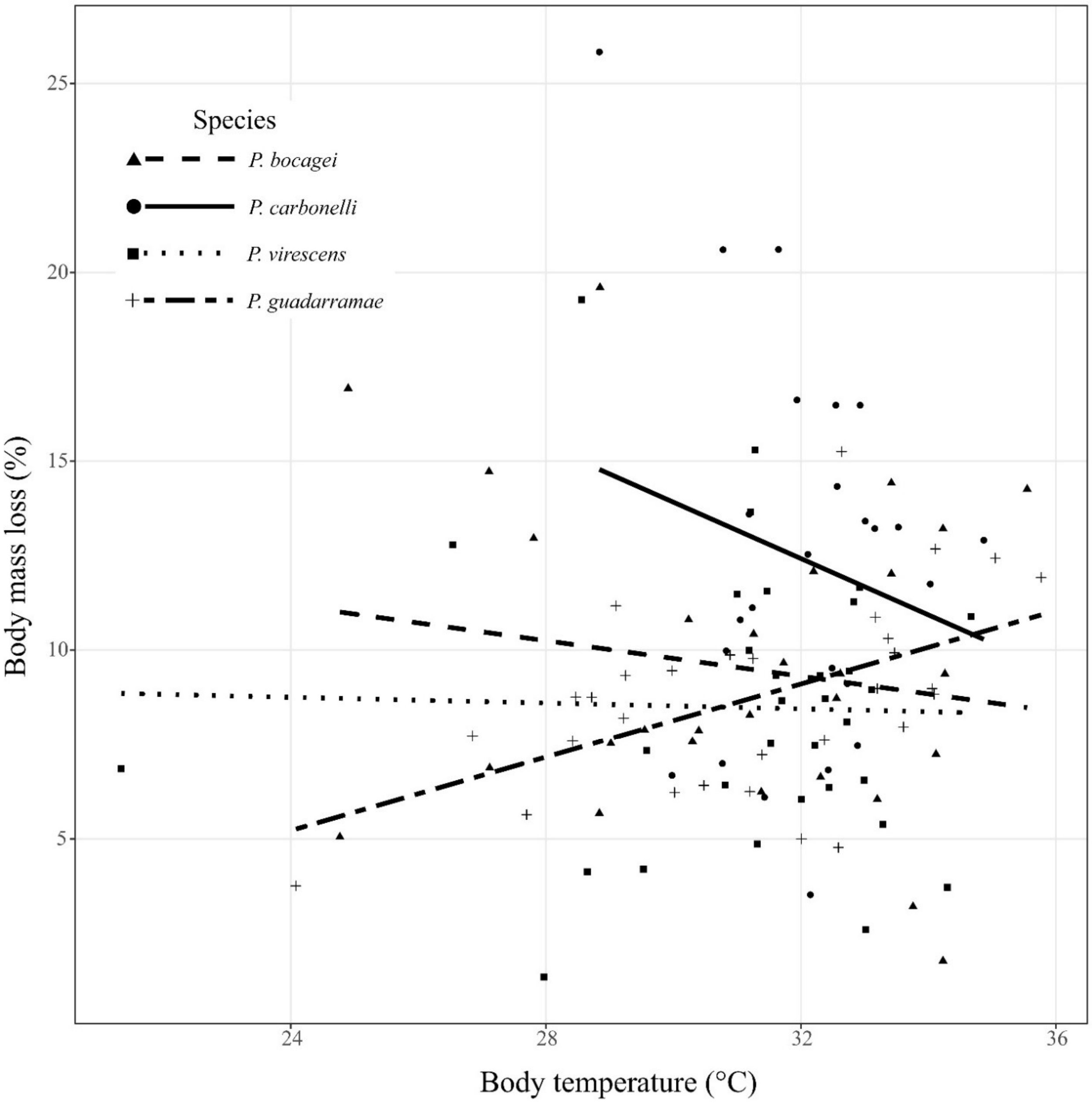

Fig. 6.1.5. The species-specific relationship between preferred body temperature and water loss corrected for individual weights.

Although this relationship is statistically non-significant for all species (P = 0.37), there is a significant difference in slope for *P. bocagei* and *P. guadarramae* (t-value = -1.985, P = 0.0497, d.f. = 108), a pair of sympatric species. This result suggests not only that individuals selecting higher body temperatures do not necessarily loose more water, but also that dehydration may impose species-specific constraints.

4. Discussion

The study of thermoregulation, water balance and their interaction are of primary importance for a better understanding of how ectotherm species interact with their environment (Huey 1982; Angilletta 2009). Research on ecophysiology becomes even more compelling when one considers the evidence on how climate change is affecting ectotherms distribution and survival (Parmesan and Yohe 2003; Parmesan 2006; Huey et al. 2009; Sinervo et al. 2010). Our results show that dehydration may have negative consequences on ectotherms' thermoregulation, leading to reduced body temperature, increasing skewness in body temperature distribution, changing the hourly pattern of thermoregulation and the use of space, and possibly imposing a physiological choice between thermoregulation and water balance.

Effects of dehydration on preferred body temperature

Despite having the same thermal availability, dehydrated lizards were, on average, colder than hydrated ones and such difference may affect several aspects of lizards' ecology. Lower body temperature may be detrimental to the ectotherms' whole-individual performance as feeding, locomotion, reproduction, social interactions, as well as predator avoidance, all depend on the maintenance of optimal body temperature (Avery et al. 1993; Selong and McMahon 2001; Angilletta et al. 2002a; Berger et al. 2008). We thus speculate that in nature, dehydrated lizards may face negative consequences concerning development, survival, and reproduction. Indeed, it has already been shown that lizards experiencing droughts are less active than those exposed to milder conditions (Ryan et al. 2016).

Dehydrated lizards also showed an increased skewness in body temperature distribution (Fig. 6.1.2). Left-skewness in ectotherms body temperature is a well-known phenomenon, that is expected for several reasons, like asymmetry in operative temperatures and thermal performance curves (Huey and Pianka 1977; Hertz 1992; Dell et al. 2011). More broadly, skewness is an intrinsic feature of physiological processes, since they increase exponentially with temperature (DeWitt and Friedman 1979). Recently, an extensive review of skewness in several desert lizard species concluded that left-skewness is common across geography and phylogeny and does not correlate with body size or median body temperature (Huey and Pianka 2018). Our results reinforce such a view, and the four species we tested reacted in a similar way to water deprivation (Fig. 6.1.2). Heavily left-skewed body temperature distribution was mainly caused by the time they spent in unfavorable thermal conditions (especially hiding inside the refuge). Thus, in the present case, dehydration directly affected the lizards' thermoregulation processes, by limiting their ability to achieve and maintain their preferred body temperature.

Change in daily pattern of thermoregulation

The second result of this study concerns the daily pattern of thermoregulation. Lizards were able to keep high body temperature throughout the day as long as dehydration was not a limiting factor. The same individuals, however, selected lower body temperatures when dehydrated. All species kept lower body temperatures at the beginning and the end of the day, while they raised their body temperature during the middle part of the day. Even at their maximum, body temperatures of dehydrated lizards were always lower than those of hydrated lizards. Since lizards spent only one day in the lab before experimentation (with access to natural light), they were still synchronised with circadian rhythm. This result could thus reflect an attempt to thermoregulate only during the hottest part of the day when perhaps the probability of finding preys (and thus replenish water) is the highest. Such a strategy would be related to the concept of hours in restriction of activity (Sinervo et al. 2010), postulating that lizards exposed to extremely high environmental temperature will be forced to reduce their activity to dawn and sunset. Our results, however, suggest that lizards tried to maximise thermoregulation during the central part of the day. For ectotherms living in temperate areas experiencing summer droughts, this strategy may conflict with excessively high environmental temperature. Indeed, during summer, many species of European lizards shift to a bimodal pattern of activity (Foà et al. 1992; Adamopoulou and Valakos 2005). We thus suggest that, on a daily basis, thermoregulation and water balance may conflict, likely because of the complex interaction between feeding behaviour and physiological balance of heat and water. In extreme cases, such conflict can lead to aestivation, the total interruption of summer activity (Pollo-Mateos and Pérez-Mellado 1989).

Dehydration constrains the use of space

In the gradients, lizards had access to a wide range of available temperatures and a refuge. In both treatments, lizards spent most of their time in the position that offered the best spot for thermoregulation. The main difference was reflected in the use of gradients extremes, hot and cold. Dehydrated lizards were more prone to use the refuge, while fully hydrated ones spent more time under the lamp. A similar result was found in *Sceloporus* lizards, which buried more often when dehydrated (Crowley 1987). Such behavioural differences may result from the attempt of dehydrated lizards to both thermoregulate and avoid water loss. Indeed, even under dehydration, lizards still selected the best spot for thermoregulation, but it is likely that they evaluated the water loss more carefully and retreated more often. A possible alternative explanation is that dehydrated lizards had lower temperature because were searching for water instead of thermoregulating. Our results do not support such a hypothesis, since dehydrated lizards most of the time selected the same spot fully hydrated ones or retreated. Interestingly, when fully hydrated, lizards used the hottest spot in the gradients more than expected. This behaviour may maximise the heat intake per a given unit of time in a situation in which individuals could indefinitely replenish the water lost through evaporation. This speculation offers a hypothesis testable in the field. Lizards subjected to higher water stress should refrain from reaching a high body temperature or from maintaining it for prolonged periods of time. Other ecological consequences of a reduced availability of suitable habitats may involve increasing intraspecific competition and limited opportunities to find food items and mates.

Thermoregulation is compromised by dehydration

Combining the evidence collected, lizard thermoregulation is compromised by water balance. When lizards had full access to water and dehydration posed no restrictions, they optimised thermoregulation, kept a constant body temperature throughout the day, and basked even in the hottest part of the gradients. Optimizing water balance would instead imply refraining from activity and retreating to the coolest spot available since in reptiles both activity level and body temperature determine the rate of water loss (Mautz 1980). Dehydrated lizards, however, did not refrain from activity, nor selected the lowest body temperature possible. Instead, they employed a mixed-strategy, thermoregulating at lower temperatures and more often seeking shelter in the refuge. Why dehydrated lizards did not maximize water balance? We speculate that total inactivity would not represent a solution for dehydrated lizards. Instead, a mixed strategy of thermoregulation and retreat may offer more chances to find prey items or free-standing water, and other lizard species show increased activity when rainfall occurs after extended dry periods (Rutherford and Gregory 2003). Such a strategy, however, may be inadequate under chronic water shortage, such as during European summer droughts (Rahmstorf and Coumou 2011).

Dehydration elicited behavioural responses that appear to be qualitatively similar in the four species we tested. All species decreased the mean body temperature, levelling on similar values. Even the sympatric pair *P. bocagei* and *P. guadarramae*, that differed in preferred body temperature when hydrated, showed no difference under dehydration. Similarly, all species showed a linear or nearly linear trend in daily thermoregulation when fully hydrated but switched to a parabolic trend when dehydrated and retreated more often in the refuge when dehydrated. However, some species differed in the slope of the regression between the preferred temperature and the body mass lost. In particular, *P. bocagei* and *P. guadarramae* showed a significant difference in their slopes, suggesting that the negative effects of dehydration on thermoregulation are species-specific. Indeed, while in *P. guadarramae* the loss of water was positively associated with preferred body temperature, the reverse was true for *P. bocagei*. This result reinforces recent findings suggesting that in lacertid species water balance may interact with thermoregulation (García-Muñoz and Carretero 2013; Osojnik et al. 2013; Sannolo et al. 2018).

Dehydration and thermal preference estimation

Our results also bear some implications for the quantification of thermal preferences under laboratory conditions, even though it should be considered that our experiment lasted overall three days—with one day of water deprivation—instead of the typical single one. Usually, lizards being tested in thermal gradients are not provided with water, because some definitions of thermal gradients stated that they should provide an environment free from ecological costs and constraints (Porter and Gates 1969; Huey and Slatkin 1976). Few authors justified the choice to not provide water if the studied species is insensitive to water loss in the short term (Rusch and Aniglletta 2017). We believe that such awareness is still rare and given the results presented here, we caution a careful approach. If the thermal preferences of the studied species are entirely unknown, or if its ecology, physiology, or distribution suggest that it may be sensitive to water loss, providing water in the gradients should be considered. Otherwise, a waterless gradient would violate the assumption that it should be cost-free environment. Furthermore, collected data on thermal preferences, although internally coherent, might be left-skewed due to the interaction between thermal preference and dehydration, and might underestimate preferred temperature.

Some practical aspects may limit the insights that this study provides about the effect of dehydration on thermoregulation. Firstly, we assumed that lizards were fully hydrated at the beginning of the experiment since they had had full access to an unlimited source of water. However, we cannot prove this assumption. Thus, our estimate of water loss reflects the percentage of body mass that each lizard lost under dehydration. Secondly, the effects of dehydration, as quantified in laboratory conditions, may be different under natural conditions. For instance, air temperature and humidity were kept constant in this study and did not fluctuated as they do naturally under circadian rhythms. Thirdly, we focused on the short-term effects of dehydration, and the results of this study cannot be readily extrapolated to infer chronic (e.g., seasonal or annual) stress. Finally, for practical reasons, we investigated the use of space in a confined setting that by necessity is an oversimplification of a natural environment. We believe, however, that such limitations did not prevent us to accurately measure how dehydration affects thermoregulation in lizards.

Conclusions

The present study shows that acute dehydration negatively affects thermoregulation in lacertid lizards. Poor thermoregulation may have profound consequences on survival, fitness, and life-history traits in ectotherms (Lorenzon et al. 1999; Angilletta et al. 2002a; Wang et al. 2016). We demonstrated here that dehydrated lizards are forced to select suboptimal body temperature, thermoregulate less precisely, and change the use of space. Available data on recent climatic scenarios, as well as predictions on future ones, indicate not only a trend in rising temperatures (IPCC 22014) but also an increase in the frequency of extreme heatwaves and droughts, at least for specific regions, like Europe (Schär et al. 2004; Dai 2013). Temperate reptiles, like the lacertid lizards used in the present study, may be particularly vulnerable to such shortages in water availability since they appear to cope poorly with such climatic extremes (Ryan et al. 2016). Future research should pose considerable attention on the short and long-term effect that water shortage may impose on wild populations of ectotherms.

Supporting Information

Table S6. Skewness values for both treatments and all species used.

Table S7. Interspecific comparison of thermal preferences for fully hydrated lizards.

Fig. S6. Boxplot of the environmental temperatures available to lizards inside the gradients.

Table S8. Mean and variance of the environmental temperatures inside the gradients

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6.2 Article VI: Variation in field body temperature and resistance to dehydration along an environmental gradient in a diurnal ectotherm

***In preparation*.**

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Abstract

Body temperature has profound consequences on all aspects of ectotherms life-history, like fitness and performance, and has been the subject of research for decades. In reptile ecophysiology, comparatively less is known on the role of water balance. Indeed, in recent years several recent studies are pointing out the importance of studying thermoregulation and water balance within the same framework. Here we used a Mediterranean lizard, *Psammodromus algirus*, to investigate how field body temperature and resistance to water loss may vary among populations and along an altitudinal gradient. We found little variation in field body temperature among populations, while within each population, field body temperature may change daily, seasonally and differ between sexes. On the contrary, resistance to water loss increased with altitude but showed no seasonal trend or difference between sexes. Microclimatic data indicated that highland lizards experienced more extreme conditions, that may enhance their resistance to water loss. Other factors, like inter-population differences in parasite intensity may also contribute in explaining the observed patterns. We present here the first data for a lacertid lizard on the inter-population variability and sexual difference in resistance to water loss and point out that water balance may play a fundamental role in regulating lizard activity during the hottest and driest period of the year.

Keywords: thermal ecology, water balance, evaporative water loss, climate change, Psammodromus

1. Introduction

Temperature has deep effects on every facet of ectotherms biology, including fitness and performance (Angilletta, 2009). Ectotherms can regulate their body temperature using behavioural adjustments (Stevenson, 1985), physiological mechanisms, and by changing the daily and seasonal patterns of activity (Adolph and Porter, 1993). Ectotherms can change their body temperature by exposing to or retreating from direct or indirect solar radiation (Cossins and Bowler, 1987), and body temperature is often kept within often narrow species-specific ranges (Cowles and Bogert, 1944; Cossins and Bowler, 1987). Thermoregulation is thus used to optimize performance (Angilletta Jr., Niewiarowski and Navas, 2002) and maximize fitness (Andrews and Schwarzkopf, 2012), by achieving and maintaining optimal temperatures for digestion (Van Damme, Bauwens and Verheyen, 1991), locomotion (Angilletta, Hill and Robson, 2002), reproductive output (Van Damme et al., 1992) and to avoid predators (Christian and Tracy, 1981).

While the ecological and adaptive significance of thermoregulation has been the subject of studies for decades (Cowles and Bogert, 1944; Licht et al., 1966; Huey, 1982; Angilletta, 2009), comparatively less is known on the role of water in reptile ecophysiology at least compared to other ectotherms like amphibians. Water balance, dehydration status or access to water are often used as modulatory factors to explain patterns in reptile life-history, ecology and behaviour. For example, restriction in water availability may constrain growth rate (Lorenzon et al., 1999), modulate habitat selection (Neilson, 2002), alter immune function (Moeller, Butler and DeNardo, 2013), and even trigger mother-offspring conflicts (Dupoué et al., 2015a). In a few cases, morphological features are used to reduce dehydration (Davis and DeNardo, 2007) or to exploit water from non-conventional sources, like in the case of rain-harvesting species (Bentley and Blumer, 1962). Still, some research areas, particularly regarding the potential interaction between thermal and water ecology remain mostly unexplored.

Indeed, several recent studies are pointing out the importance of studying thermoregulation and water balance within the same framework. For example, it has been shown that lizards' thermoregulatory activity may be affected by the short-term rainfall regime (Ryan et al., 2016) and dehydration status (Sannolo et al. in review). Similarly, while their thermal preferences may be similar, lizard populations that have access to limited free-standing water may be more resistant to water loss than those population with unrestricted access to water (Dupoué et al., 2017). However, the research on the ecological, physiological and adaptive role of water in reptiles, and especially in lizards, still lacks behind if compared with the information available on thermal biology. For example, it is mostly unknown to what extent resistance to water loss may vary across environmental gradients, or if it is subject, like thermal preferences often are, to daily and seasonal plasticity.

The large psammodromus, *Psammodromus algirus*, is a medium-sized lacertid lizard (SVL up to 93 mm; Fig. 6.2.1), widely distributed in Mediterranean environments of the Iberian Peninsula, SW France, and in NW Africa (Sindaco and Jeremčenko, 2008). This lizard species serves as an excellent case study for the present study because while its thermal ecology has been studied extensively, scarce information is available on the potential variability of its resistance to water loss. The large psammodromus prefers high body temperatures if compared with most other lacertid species. Its preferred temperature, measured in standard thermal gradients, is around 35.6°C (Díaz, 1997). In the field, body temperatures ranged 30-33°C, and it is subjected to daily and seasonal variation (Carrascal and Díaz, 1989; Diaz, Iraeta and Monasterio, 2006). This species thermoregulate carefully by selecting the appropriate microhabitat and heat source (Belliure and Carrascal, 2002). Little variability in selected body temperatures was detected along altitudinal gradients (Diaz et al., 2006; Zamora-Camacho, Reguera and Moreno-Rueda, 2016). This species is polygynic and males are strongly territorial. As a result, inter-sexual differences in body temperature may result as a consequence of reproductive behaviour (Diaz, 1993; Iraeta, Salvador and Diaz, 2012). On the contrary, scarce information is available on resistance to water loss and none on the potential variability among populations or across environmental gradients. Only one work reported the amount of water loss at low body temperature (24°C) throughout a day from a single locality (Ferreira, Santos and Carretero, 2016).

The aims of the present work were multiples. (1) Field body temperatures and resistance to water loss may both varies depending on the altitude. Previous evidence suggested that the thermal ecology of the large psammodromus is somewhat conservative and insensible to changes in altitude (Diaz et al., 2006; Zamora-Camacho et al., 2016). Hence, we would expect to find no significant variation in body temperature across altitudes. Instead, for lizards, there is no information on the potential variability of resistance to water loss across along an altitude gradient. If along the altitudinal gradient sampled in this study air temperature decreases with altitude while humidity increases with altitude (Körner, 2007), we would expect to find a pattern of higher resistance to water loss in lowland populations, if lizards are acclimatised or adapted to local meteorological conditions. On the contrary, high-altitude populations should be less resistant to water loss. (2) We expected that both field body temperatures and resistance to water loss to vary according to daily and seasonal patterns. Previous research reported both daily and seasonal plasticity in lizards' field body temperature (Diaz et al., 2006; Zamora-Camacho et al., 2013), while no data is available on the potential variability of resistance to water loss. (3) Finally, we would expect to find inter-individual differences in field body temperature and resistance to water loss, especially when the comparison is made on body size (small versus large individuals) and between sexes (Diaz, 1993; Diaz et al., 2012).

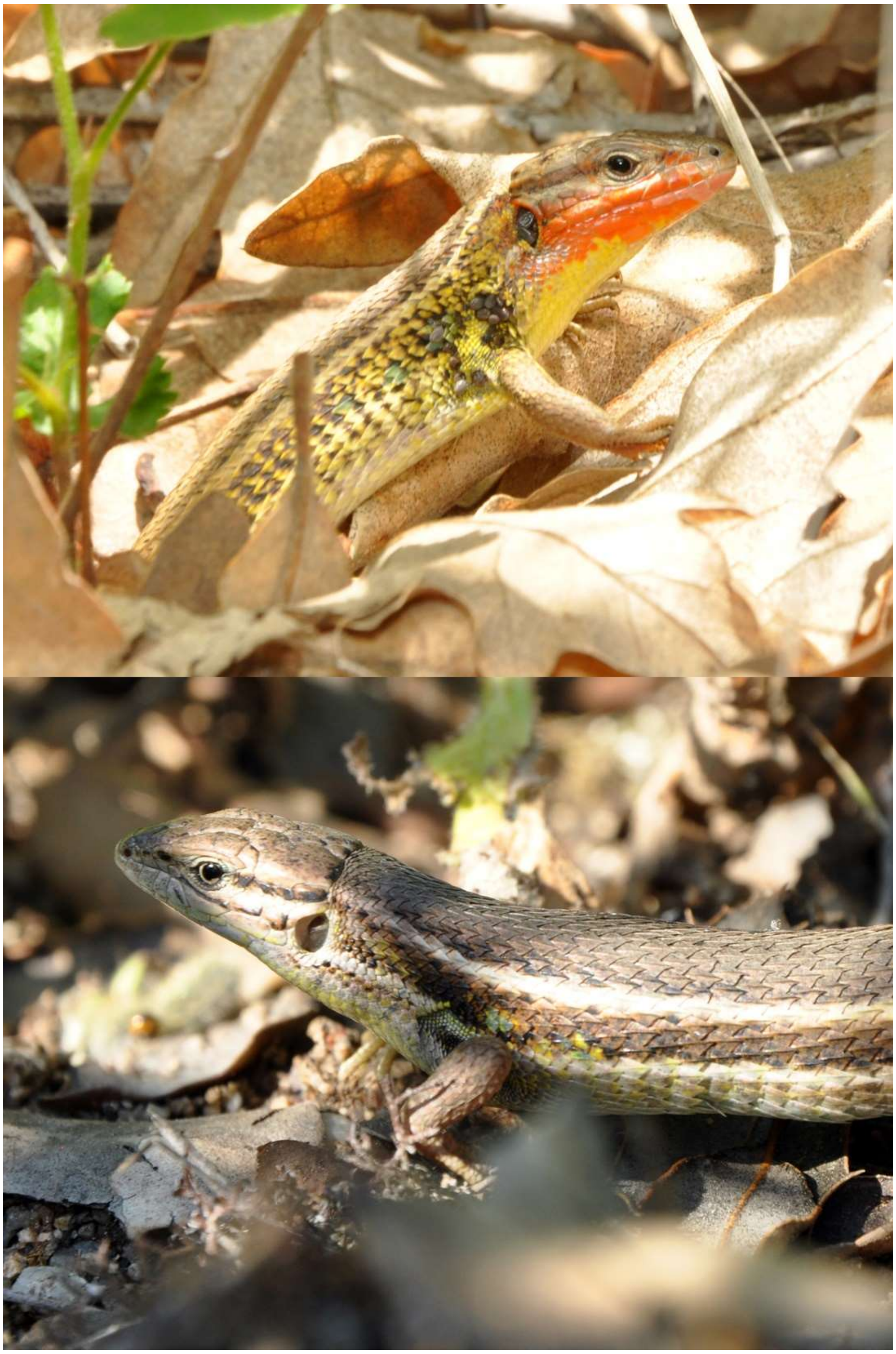

Fig. 6.2.1. An adult male (top) and an adult female (bottom) of *Psammodromus algirus* used in this study. The male belonged to the highland population, while the female came from the lowland population. While the female showed no ectoparasite, note the high number of ticks parasitizing the male. The prevalence and intensity of ticks is higher in the highland population than in the in lowland population.

2. Methods

2.1 Study sites

We sampled three populations, distributed along an altitudinal gradient (650-1300 m.a.s.l.) approximately 40 km long. The lowest altitude location, “Monte de El Pardo” (abb. El Pardo; 40°30’N, 3°45’O), is situated close to the city of Madrid. It is characterised by sandy soil and open *Quercus ilex* woodland. Lizards are mainly found within the undergrowth composed by oak sprouts and bushes of *Cistus* sp. The mid-altitude location, “Puente Medieval de Colmenar Viejo”, (abb. Colmenar; 40°40’N, 3°49’O; mean 850 m.a.s.l.) is situated at midway between the other two locations (20 km from El Pardo and 20 km from the Guadarrama range). It is characterised by a sandy clay loam substrate, with abundant rock outcrops. Tree cover is mostly composed by sparse *Juniperus oxycedrus* and few *Quercus ilex*. Lizards are mainly found close to oak sprouts or in the bush matrix of *Thymus*, *Lavandula* and *Festuca* sp. Finally, the high-altitude location is at the base of the Sierra de Guadarrama mountain system (abb. Navacerrada; 40°43’N, 4°01’O; mean 1250 m.a.s.l.). The tree coverage is composed almost entirely by *Quercus pyrenaica*, while the undergrowth is dominated by *Cistus* sp. Lizards are found mostly at the ground level within the matrix of rock rose bushes. The lizards from these populations belong all to the same haplogroup and showed significant gene flow among them (Díaz et al., 2017).

2.2 Sampling, measuring and husbandry

Adult lizards (SVL > 60 mm) were searched by eye and captured by noosing in the three areas during their activity time (~9:00-18:00) in the reproductive season in April and May 2018. Within a few seconds from capture, the cloacal temperature was measured using a thermometer fitted with a K-thermocouple probe (Hibok 18, precision: 0.1 °C, accuracy: ± 0.2%) within less than 15 s since the capture. For each lizard, we also recorded the time of capture and the GPS position. Lizards were brought to the “El Ventorrillo Research Station” where small groups (max five lizards of the same sex) were housed in large plastic terraria (~70×40×40 cm) divided by locality. Each terrarium had two sources of water, two shelters, and access to natural light. Measurements of Total Evaporative Water Loss (TEWL) were carried on after one day of resting, during which we assumed that lizards re-hydrated if necessary. After TEWL measurements we sexed according to sexual secondary characters (Mellado and Martínez, 1974) and measured the SVL (with a calliper to the nearest 0.01 mm) of each lizard and then released them back at the site of capture. Lizards did not spend more than five days in captivity. Water was provided ab libitum, while food in the form of Tenebrio molitor larvae was provided every day only after the TEWL measurements.

2.3 Avoiding pseudoreplication

Pseudoreplication (Hurlbert, 1984) may impinge the assumption of independence of the data and undermine the results of a study. In this work, we sampled each population several times. Hence, we needed to avoid sampling the same individual more than once. We adopted several precautions, both while sampling in the field and during the analysis of the data. First, in each site we sampled over a large area, and along different transects. Second, when each lizard was released, it was also stained on the neck with white nail varnish, that in some individuals lasted up to 14 days (pers. obs.). Finally, we photographed dorsally, laterally and ventrally each lizard. Photo-identification has already been applied several times to lizards (Sacchi et al., 2016) and *P. algirus* has conspicuous features, like lateral blue ocelli variable in number and size, that enables the researcher to distinguish among individuals. In the case of doubtful individuals, the cross-check of location, sex, body size, and picture comparison enabled us to reduce to the minimum the risk of using an individual more than once.

2.4 Microclimate measurements

In each sampling area, we placed 12 iButton Thermochron®, evenly spaced on transects of approximately 1 km long (Model DS1923; Maxim Integrated Products, Sunnyvale, CA, USA). Dataloggers recorded temperature every hour, and six in each sampling area also recorded humidity. We placed the dataloggers in two different habitat types: under rocks, in full shade, and on open ground, in full sun. We believe that such microhabitats encompass the whole spectrum of temperatures and humidity that lizard may experience in the field. Because the gathered data are inevitably temporally autocorrelated, we summarised the data by pooling together each day measurements.

2.5 Water loss measurements

Total Evaporative Water Loss (TEWL) was measured as the individual difference in body mass following the protocol of Sannolo et al. (2018). Briefly, we first weighted each lizard to the nearest 0.001 g with a precision balance (Sartorius M-Pact AX224; Sartorius AG, Goettingen, Germany). We then set a thermal chamber (FRIOCELL FC-B2V-M/FC-404, accuracy at 37°C ± 0.3 °C, MMM group Germany) at 35°C to mimic the preferred temperature of this species (Díaz, 1997). Each lizard was then placed inside a cylindrical plastic box (11 × 10 cm) provided with holes on all sides. The boxes were placed in the incubator in random order and position with respect to sex and location. The incubator was sealed and after nine hours (9:00-18:00 h) we measured each lizard again to determine TEWL.

2.6 Data Analysis

We first tested for potential differences in body size and body condition among populations for each sex separately. Differences in SVL were calculated using one-way ANOVA with the population and sex as fixed factors. We checked that the residuals of the model followed a normal curve (Shapiro-test = 0.99, P = 0.64). We calculated body condition as the residuals of the model II regression of the log SVL on the log body mass (Green, 2001). The residuals followed a normal curve (Shapiro-test = 0.99, P = 0.16).

Before modelling field body temperature and resistance to water loss, we checked the deviation from normality of these dependent variables. Field body temperature was slightly left-skewed (-0.82), as it is usually the case in lizards (Huey and Pianka, 2018). EWL expressed as a percentage was right skewed (0.42). Because water loss was negatively correlated with body size (t-value1,169 = -4.583, P < 0.0001), all analysis involving water loss used percentages instead of the raw data. We then used the R package fitdistrplus to plot a Cullen and Frey graph to find the appropriate distribution curve for these variables. AIC comparisons indicated that EWL best approximates a gamma distribution, while field body temperatures reasonably fitted a normal curve. Hence, we used a linear model to test for the effect of altitude, time of the day, sex and day of the season on filed body temperature (formula: Body temperature ~ SVL + locality × day + locality × sex + locality × [time + time2]). Instead, we fitted a GLM model with a gamma link on the water loss data to test for the effect of altitude, the day of the season and sex (formula: EWL ~ SVL + locality × day + locality × sex).

Microclimatic data on temperature and relative humidity were filtered to retain only daytime measurements (08:00 – 20:00 h) and were averaged throughout each day. We then fitted two GLS models (one for the temperature and one for humidity data) to test the potential effect of seasonal trending, differences among localities and position of the data-logger (formula: temperature (humidity) ~ day × locality × datalogger position). We included in the model an autocorrelation structure to account for the temporal autocorrelation of the data. Finally, we summarised the daily maximum and minimum of both temperature and humidity for each location and computed the range of fluctuation of both types of data. All statistical analysis was run in the R environment (R Development Core Team, 2018). Figures were produced with the ggplot2 package (Wickham, 2009).

3. Results

Over the course of 40 days we sampled 171 lizards (El Pardo n = 58; Colmenar = 47; Navacerrada = 66). SVL showed no significant variation neither among populations ($F_{2,168}$ = 1.006, P = 0.368) nor between sexes ($F_{1,165}$ = 2.368, P = 0.123). Similarly, body condition was no different among populations ($F_{2,168}$ = 0.954, P = 0.387), but differed between sexes, with males being heavier than females for a given SVL ($F_{1,165}$ = 59.53, $P < 0.0001$). Hence, the following results were not due to potential differences in body size or body condition among populations. Overall, field body temperature showed no significant variation along altitude (P = 0.502; El Pardo = 31.6 ± 2.7°C, Colmenar = 32.5 ± 1.9°C, Navacerrada = 31.6 ± 2.1°C; Fig. 6.2.2). However, field body temperatures showed significant variability depending on sex, time of day and day of study. Sexes differed in body temperature in both the lowland and highland populations (El Pardo t-value = 2.753, P = 0.0064; Navacerrada t-value = 2.506, P = 0.0133; Fig. 6.2.2).

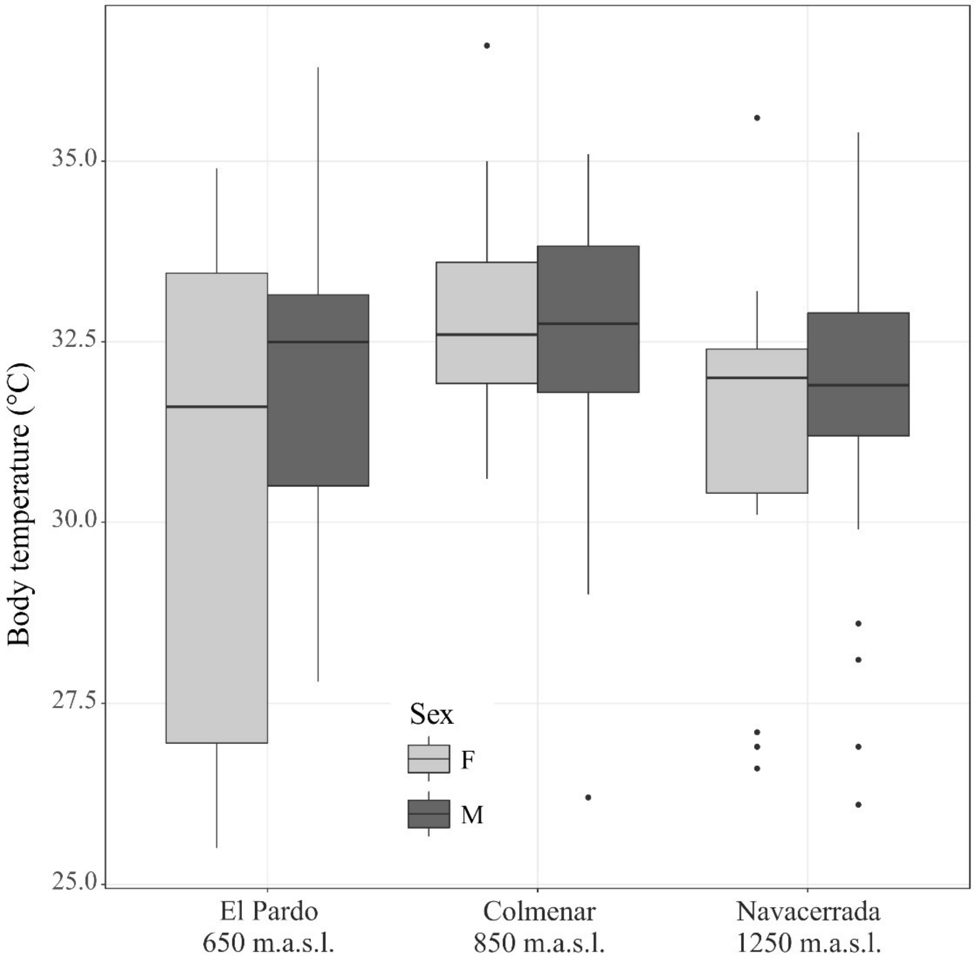

Fig. 6.2.2. Boxplot of field body temperatures along altitude and between sexes.

Sexual differences in field body temperatures varied depending on the population. Indeed, while in the lowland population males had higher body temperatures than females (t-value = 2.753, P = 0.0066), the reverse was true for the highland population (t-value = 2.506, P = 0.013). Instead, sexes showed no difference in body temperature at midland (t-value = -0.894, P = 0.37). Field body temperatures significantly decreased along the day in the lowland population, while showed no trend in the other two populations (El Pardo t-value = -2.179, P = 0.031; Supplementary material, S1). Body temperatures also increase throughout the season in the lowland and highland populations (El Pardo t-value = 5.321, P < 0.0001; Navacerrada t-value = 4.355, P < 0.0001; Supplementary material, S2).

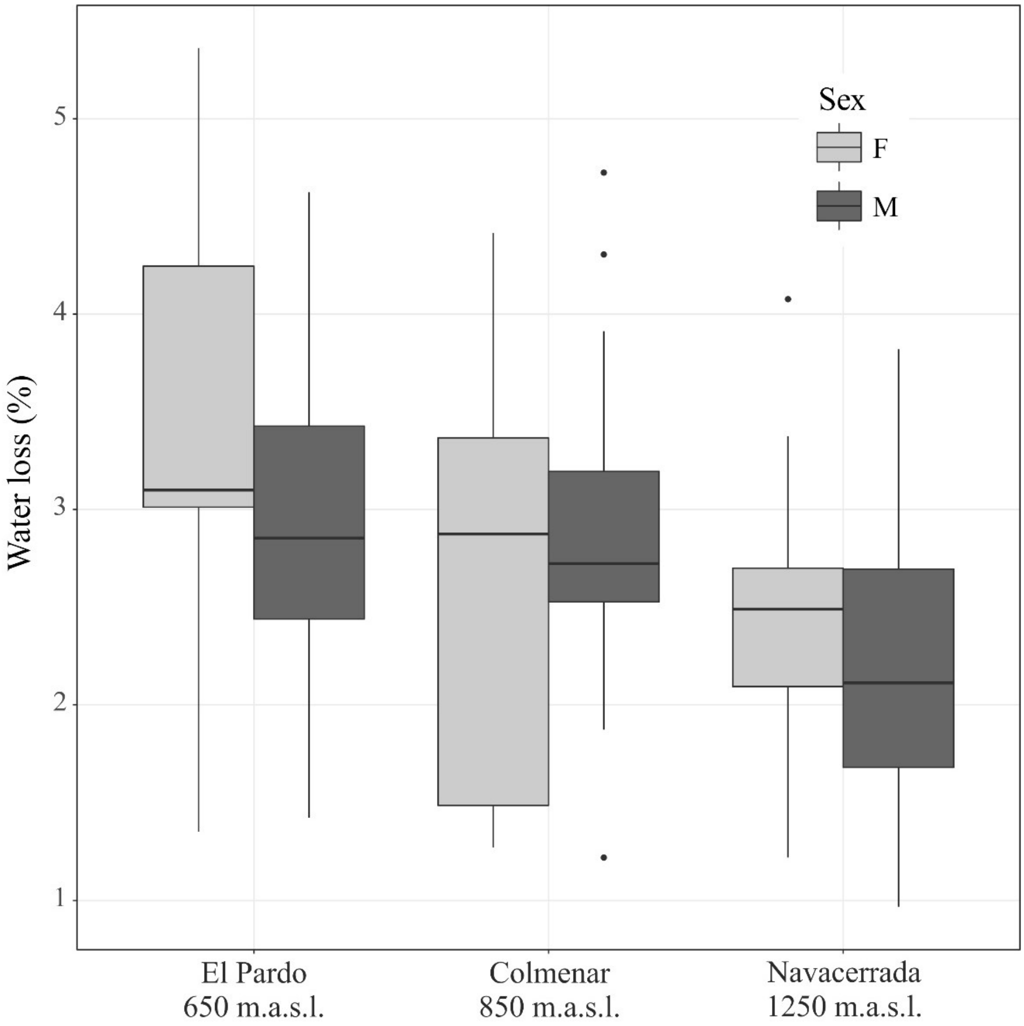

Fig. 6.2.3. Boxplot of total evaporative water loss along altitude and between sexes.

Resistance to water loss, unexpectedly, increased with altitude, with lizards from the highland population losing proportionately less water than lizards from the lowland population (t-value = 5.393, $P < 0.0001$; El Pardo mean 3.08 ± 0.9 %; Colmenar = 2.81 ± 0.92 %; Navacerrada = 2.27 ± .0.71 %; Fig. 6.2.3). However, the resistance to water loss appeared to be more conservative than field body temperatures in other aspects. Indeed, EWL did not change throughout the season in any population ($P > 0.05$; Supplementary material, S3), and we did not detect significant differences between sexes within any populations ($P > 0.05$; Fig. 6.2.3).

The dataloggers showed that temperatures measured in shade were on average lower than those in full sun for all localities (El Pardo t-value = 2.449, $P = 0.0150$; Colmenar t-value = 2.946, $P = 0.0035$; Navacerrada t-value = 2.548, $P = 0.0115$). Conversely, humidity in shade was on average higher than in full sun (Colmenar t-value = -5.278, $P < 0.0001$; Navacerrada t-value = -6.675, $P < 0.0001$). We did not detect significant trends in temperature during the study, either in shade or sun ($P > 0.05$ for all localities; Fig. 6.2.4 and Fig. 6.2.5). Similarly, humidity measured in shade did not show significant trend during the study ($P > 0.05$ for all localities; Fig. 6.2.4 and Fig. 6.2.5), while humidity measured in full sun increased significantly in the lowland and highland populations (El Pardo t-value = 2.228, $P = 0.0268$; Navacerrada t-value = 4.318, $P < 0.0001$). Comparisons among localities showed that temperature did not significantly change with altitude neither when measured in the shade nor in full sun ($P > 0.05$ for all comparisons; Fig. 6.2.4 and Fig. 6.2.5). Humidity, instead, when measured in shade, was lower in lowland if compared to the other two locations (El Pardo vs. Colmenar t-value = 2.638, $P = 0.0089$; El Pardo vs. Navacerrada t-value = 3.122, $P = 0.0020$; Fig. 6.2.4). Instead, humidity measured in full sun showed no significant variation across localities (for all comparisons; Fig. 6.2.5)

When we focused on extremes instead of average values, we found that the highland locality experienced both the lower minimum and higher maximum in both temperature and humidity in full sun. Similarly, in full shade, the highland locality experienced broader fluctuations in temperature (Table 6.2.1). The ranges of temperature both in shade and full sun increased with altitude, while the ranges of relative humidity decreased with altitude in shade but showed no pattern in full sun. For all localities and both temperature and humidity, the amplitudes of extremes were higher for measurements made in full sun respect to those made in full shade (Table 6.2.1). Hence, for example, lizards in El Pardo experienced in full sun a day whose mean temperature was over 30°C but then drop to 12°C the next day (Fig. 6.2.5). Lizards in Navacerrada, instead, were exposed within two days to 24°C drop in temperature, passing from 32°C to only 8°C (Fig. 6.2.5).

	Full shade		Full sun	
	Temperature	Relative humidity	Temperature	Relative humidity
El Pardo (650)	11.0 – 20.1 (9.1)	51.7 – 98.3 (46.6)	12.1 – 30.4 (18.3)	34.2 – 96.6 (62.4)
Colmenar (850)	11.1 – 21.6 (10.5)	72.1 – 99.3 (27.2)	9.2 – 30.4 (21.2)	32.8 – 94.7 (61.9)
Navacerrada (1250)	10.1 – 22.3 (12.2)	83.1 – 100 (16.9)	8.0 – 32.0 (24)	29.0 – 100 (71)

Table 6.2.1. Minimum and maximum daytime (8:00 – 20:00 h) averaged values of temperature and relative humidity measured by dataloggers placed in full sun or full shade in the three sampling localities. Within brackets are the ranges. Temperature is express in Celsius degrees, relative humidity as % of saturated air. The number near the locality name indicates altitude above the sea level, in meters.

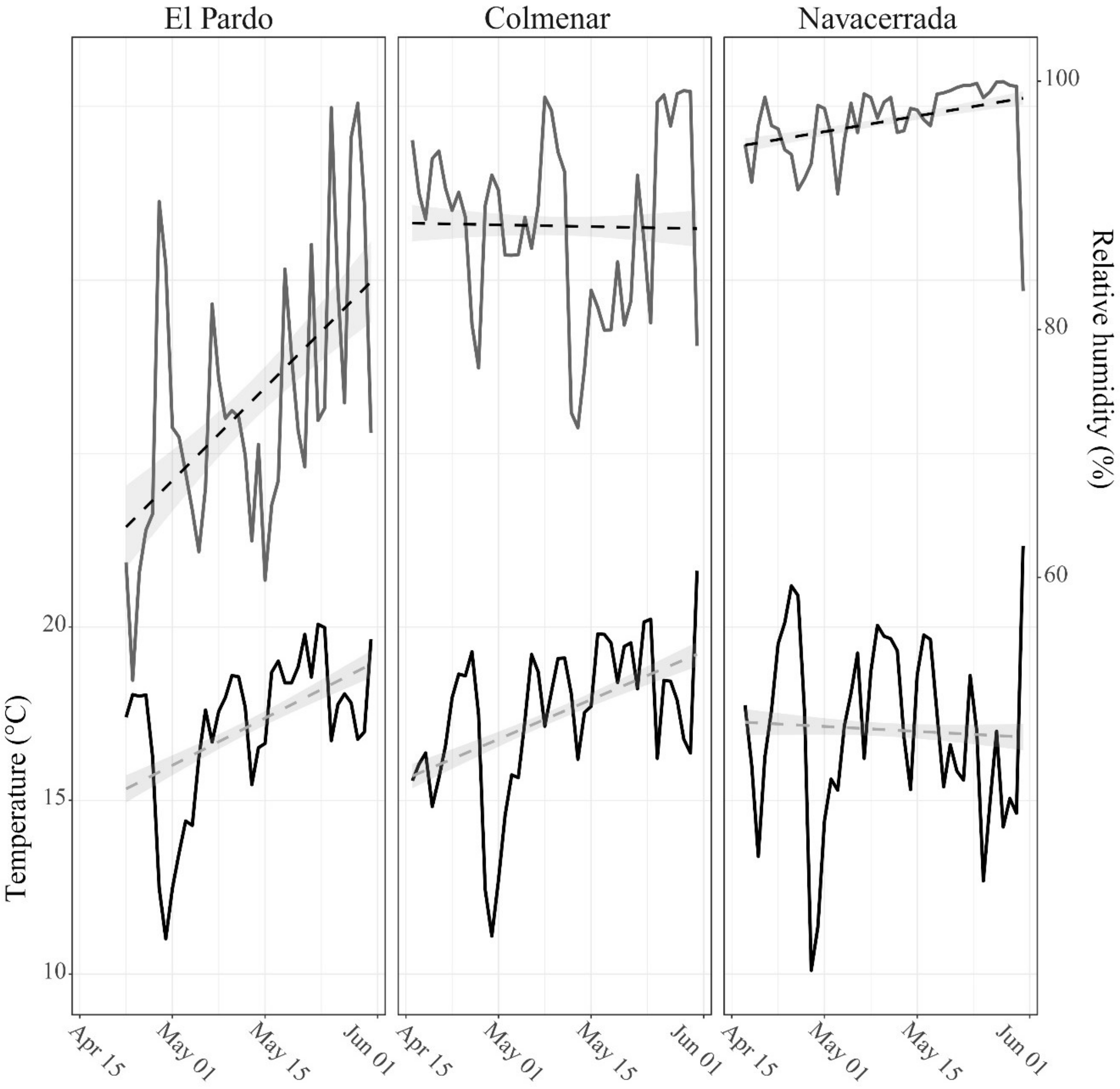

Fig. 6.2.4. Daytime temperature and humidity measured by the dataloggers in full shade. The top part shows the daily averaged humidity, while the bottom part shows the daily averaged temperature. Note that left scale refers to temperature and right scale refers to humidity.

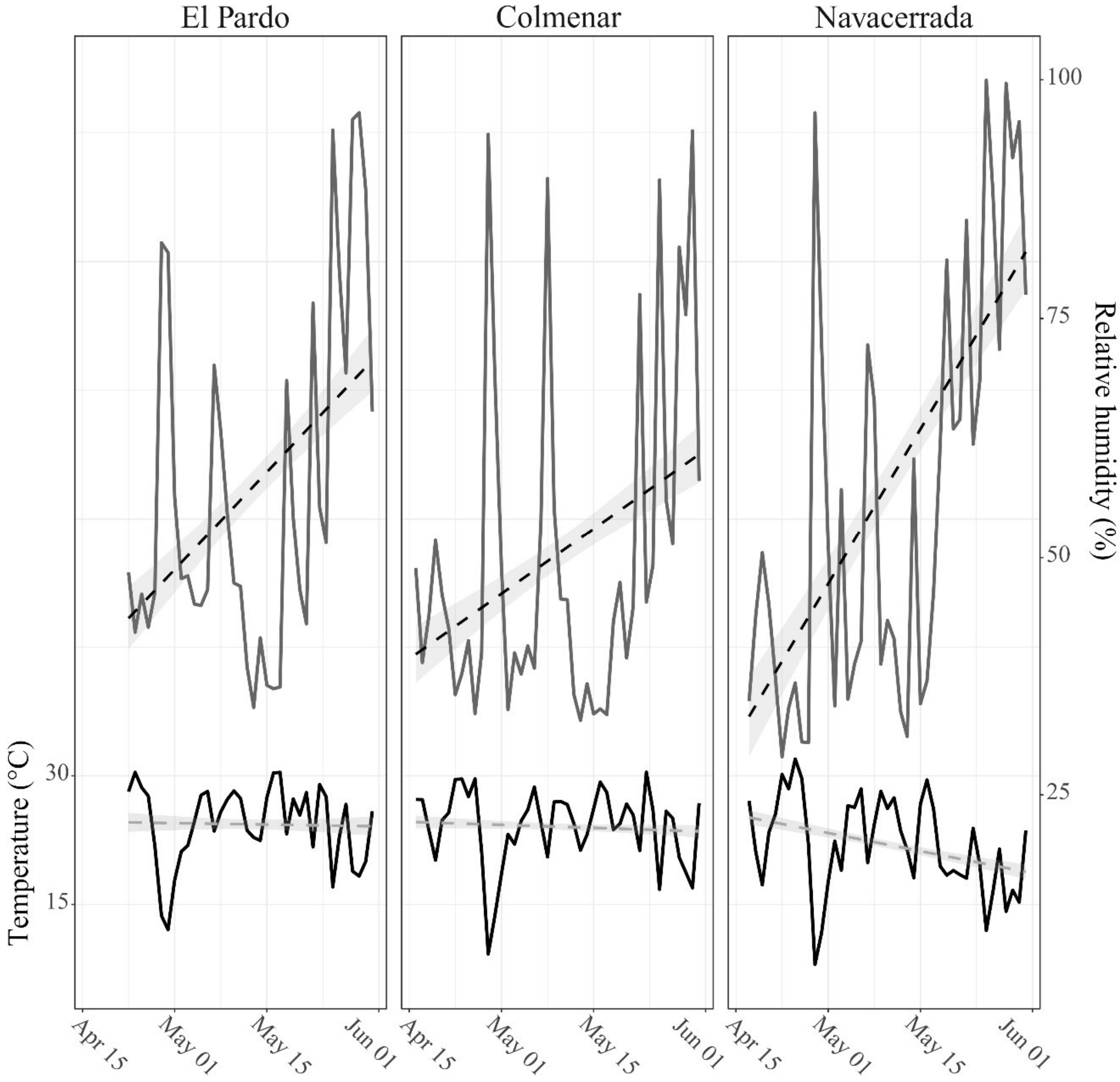

Fig. 6.2.5. Daytime temperature and humidity measured by the dataloggers in full sun. The top part shows the daily averaged humidity, while the bottom part shows the daily averaged temperature. Note that left scale refers to temperature and right scale refers to humidity.

4. Discussion

The present work aimed to investigate the inter-populational variability and potential short-term seasonal change in field body temperature and resistance to water loss along an altitudinal gradient in a diurnal lizard. We found partial support for the first initial hypotheses we formulated. Indeed, field body temperature showed no significant variation along altitude during the same time period. However, contrary to expectations, resistance to water loss increased with altitude. Similarly, we also found partial support for the second hypothesis: while field body temperatures showed daily and seasonal variability, resistance to water loss appeared to be conservative along the season. Finally, we found significant differences between sexes in field body temperature but not in resistance to water loss.

The field body temperature appeared to be constant along altitude, a result comparable to that already published for a different lineage of the same species over a broader altitudinal range (Zamora-Camacho et al., 2016; Díaz et al., 2017). Similarly, depending on the species considered, other lacertid species showed either weak or significant differences in thermal preferences among population (Van Damme et al., 1989; Artacho et al., 2013). The present study focused on a relatively small altitudinal range (~ 600 m) and dataloggers indicated that the sampling locations did not differ significantly in temperature during the study. Hence, our results may reflect either rigidity in thermal preference or the lack of variability in environmental temperature. In a previous study on the same species, however, thermal preferences of various populations were similar despite operative temperatures decreased with altitude (Zamora-Camacho et al., 2016). Hence, we inclined toward rigidity in thermal preference for this species. We found, instead, that body temperature was more flexibility on daily and season basis and depending on sex, at least for some populations. Indeed, for several lacertid species are reported daily fluctuations in field body temperature, as well as season acclimation and sexual differences (Castilla, Van Damme and Bauwens, 1999). When faced with predictable climatic fluctuations, like seasonal changes, individuals may anticipate the change and respond to it appropriately (Angilletta, 2009). In the present case, however, a significant increase in average field body temperature in two populations was not accompanied by an increase in microclimate mean temperature. Thus, it is possible that lizards increased their body temperature forecasting a typical increase in mean air temperature from early spring to summer, that was not registered during this study.

Contrary to expectations, resistance to water loss increased with altitude. Our initial hypothesis was based on the assumption that the mean environment temperature would systematically decrease, and humidity increase with altitude. Climatic variables along altitude are not always predictable, especially on a small scale and a reduced period

(Körner, 2007) and, in fact, we found significant variation only in humidity, and not in all localities and microhabitats. Still, highland lizards faced on average cooler and more humid conditions. If lizards from each population were acclimatised or adapted to the local climatic conditions, their resistance to water loss should be higher at lower altitudes, where to driving force to lose water was greater (Gates, 1980). The apparent contradiction between microclimatic and physiological measurements might be explained if we consider extremes instead of the average values of microclimatic conditions. Indeed, a recent work pointed out that extremes operative temperatures are better descriptors than averages of the thermal environment experienced by small animals (Camacho, Trefaut Rodrigues and Arturo Navas, 2015). In the present case, the high-altitude population experienced both the highest and the lowest temperatures and humidity, especially in the open. Hence, highland lizards might be more resistant to water loss because they are exposed to more extreme conditions than lowland ones. A possible, but not mutually excluding alternative, is based on a biotic factor. The lizard populations we studied show a different degree of parasitisation (Fig. 6.2.1). In particular, the prevalence of ticks is higher in lizards from the high-altitude population (Carbayo, Martín and Civantos, 2018). Ticks are typically found in groups in hotspots just behind the armpit and the neck, where their action often exposed to a variable extent the skin below the scales. Being more parasitised by ticks, lizards from the highland population may compensate by altering the superficial blood flood, and capillary structure which incidentally may reduce the water loss rate. Anatomical differences between tick-resistant and non-resistant individuals have been demonstrated at least in cattle (Schleger, Lincoln and Bourne, 1981).

Contrary to field body temperature, resistance to water loss appeared to be conservative along the study. We are fully aware that we measured different individuals across the season, and we lacked repeated measures for the same lizard. However, the same is valid for field body temperatures, which instead showed seasonal variation. Even being cautious, it seems that while field body temperature was flexible in time and less flexible along an altitudinal gradient, the opposite conclusion can be drawn for the resistance to water loss. Such a result may bear important ecological consequences. If resistance to water loss is rigid, during the summer months, water retention may constrain lizards’ activity more than hot temperatures. Indeed, a recent study showed that lizard thermoregulation during summer reflected the short-term pattern of rainfall (Ryan et al., 2016). Unfortunately, the potential for a trade-off between thermal preference and dehydration in lizards has received little attention (Crowley, 1987) and in lacertid lizards has been tested so far only in laboratory conditions (Pollo Mateos and Pérez-Mellado, 1989; Sannolo et al. in review).

As far as we know, the present work is the first in testing for sexual differences in EWL rates in a lacertid lizard. Even though our results indicate a lack of significant difference, the observed pattern suggests that females from all population lost more water than males. Such a result may indicate a lack of statistical power, as well as consistent intersexual differences of several kind, from a higher metabolic stress during the testing to morphological or physiological differences between sexes (in this species, at comparable SVL, female's trunk is longer than male's, while the male's head is bigger; Mellado and Martínez, 1974). For example, pregnant females of a viviparous snake lost more water than control snakes, likely due to the stretching of the skin caused by growing embryos. Similarly, shedding snakes lost more water than control snakes (Dupoué et al., 2015b). For lizards, no comparable data is instead available on the potential effect on EWL rates of sex, morphology or physiology.

Finally, while comparing field body temperatures across species is relatively straightforward, comparing EWL rates is challenging due to the scarcity of data and the wide range of temperature at which lizards are tested. The only published work on lacertid lizards estimating EWL rates at their preferred temperature is on two Podarcis species (Sannolo, Barroso and Carretero, 2018). At 32°C, *P. bocagei* and *P. g. lusitanicus* lost slightly more water than the large psammodromus at its preferred temperature, 35°C, over a comparable amount of time (3.3 and 2.9 % respectively). At 37°C, Podarcis lizards lost way more water than *P. algirus* at its preferred temperature (4.1 and 3.7 %). This result is not surprising, as Psammodromus lizards generally live in drier environments than Podarcis (Arnold, 1987). Furthermore, scale morphology in these two groups differs significantly, with Podarcis having small semi-beaded non-overlapping scales and Psammodromus having large, keeled and overlapping scales. Even though scale morphology in lacertid lizards has been correlated with performance and crypsis (Arnold, 2002), recent investigations on *Anolis* lizards showed that scale size and number may correlate with climatic factors like rainfall regime and aridity (Wegener, Gartner and Losos, 2014). Furthermore, even though the composition and thickness of the skin lipid layer is the main factor that determines skin permeability to water (Roberts and Lillywhite, 1980), the potential role of scale morphology in enhancing or reducing water evaporation at the boundary layer remains mostly unknown.

Concluding, we found little variation in field body temperature along an altitudinal gradient, while EWL rates decreased with altitude. We also provide here the first data on inter-population variability on EWL for a lacertid lizard. Rigidity in thermal preferences may account for the little variation observed and is congruent with previous findings on other populations of the same species, while both biotic factors like extreme temperature and humidity, as well as biotic factors, like parasite intensity, may explain the decrease in EWL with increasing altitude. The absence of variation in EWL rates along the study may suggest that during summer months water retention may constrain activity more than temperature.

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Chapter 7: General Discussion

“The most erroneous stories are those we think we know best — and therefore never scrutinize or question.”

— Stephen J. Gould (1996)

7.1 Overview

The present work, rather than focus and drill into a specific biological question, is evidently the *summa* of several investigations that tackled multiple aspects of lizard thermal ecology and water balance from various perspectives. Even though I hope I was able to link the presented works logically and to follow a coherent path, I will not hide that this thesis is the result of a personal and scientific voyage that —inevitably— was mottled of stops, rethinking, trials and errors and partial conclusions. Similarly, for several reasons, it has sometimes been hard for me to translate what I considered good theoretical and experimental questions into finished pieces of science. I guess that traveling along a PhD means also facing this kind of less obvious challenges.

Each article presented here came equipped with a discussion. Hence, I will try here to avoid repeating myself and instead discuss on a —hopefully— higher philosophical level the relevance of the results already exposed. In the first article, I laid the ground to extend the use of IR cameras in thermal studies, as this technology is still sometimes criticized because of the lack of comparison with more traditional ones like cloacal readings. In the second article, I quantified in the field, using an IR camera, the decrease in body temperature that lizards experienced while hiding, and which factors may modulate hiding behavior. The chapter 5 (article III and IV), hinged instead on quantifying and comparing the thermal ecology and water balance of closely related species that can often be found in syntopy. In article III I demonstrated that, while thermal ecology varies little —but significantly— between species, the resistance to water loss differs to a higher degree. In article IV I compared the running performance of two related species across different temperature finding a sustained thermal range over which performance was maximized and minor intraspecific differences. Finally, chapter 6 was focused on the interaction —and potential trade-off— between thermoregulation and water balance. In article V, I used several species to demonstrate that thermoregulation is impaired by dehydration. Indeed, dehydrated lizards were forced to reduce their body temperature and hide inside refuges more often than fully hydrated lizards. Finally, in article VI, I investigated the potential for inter-population variability in field body temperature and resistance to water loss along an altitudinal gradient. While body temperature was conservative across populations, it changed within population depending on the time of the day, sex and showed seasonal acclimation. Conversely, the resistance to water loss differed among populations —increased along an environmental gradient — but was conservative concerning sex and did not changed along the season.

7.2 Infrared data in Thermal Ecology

One of my first goals during the doctorate had to do with how to measure the body temperature of small ectotherms like lizards. The question may seem trivial, but only if you look at it superficially. Indeed, even without any scientific knowledge on the subject, how do you measure an animal's body temperature? Are all body parts always at the same temperature? Which tool should I use? Do I modify the animal temperature while I measure it? Can I compare —and how— thermal data gathered with different tools? And how does a specific tool measure body temperature anyway?

Indeed, the very onset of thermal ecology field studies depended on the development of thermometers small enough to be carriable in the field and thermocouples thin enough to be inserted into the reptile's cloaca (Bogert 1939; Cowles and Bogert 1944). The revolution of portable thermocouples was so widespread that Heath (1964) designed an experiment to persuade scientists to stop going around comparing air temperature against cloacal temperature to report that reptiles were thermoregulating. In more recent years dataloggers have been extensively used to record operative temperatures to assess if an ectotherm is thermoregulating (Hertz et al. 1993). Similarly, poor protocols and models in collecting operative temperature have been criticized, as even small errors may entirely impinge the relevance of the collected data (Dzialowski 2005; Angilletta and Bakken 2014).

During the last years, infrared tools partially underwent similar scrutiny. IR pistols, for example, have been repeatedly criticized for their lack of accuracy and significant errors, mostly due to the increasing surface of the laser cone with distance, and the orientation of the pistol (Hare et al. 2007; Carretero 2012). As far as I am aware of, studies using IR cameras managed to elude rigorous scrutiny so far, at least in the biological sciences. For example, IR data have been used for several years in veterinary science for diagnostic purposes (Rekant et al. 2016). However, several important works are pointing out the uses and potential misuses of IR technology (Kastberger and Stachl 2003; Vollmer and Möllmann 2018).

In reptile studies, the use of IR cameras is slowly increasing (most models are still relatively expensive), even though my feeling is that thermal ecologists are more conservative and critic about accepting data collected with new protocols or equipment. In most studies, IR data are used without a direct comparison with data collected with more traditional and widespread methodology, like thermocouples. For example, IR cameras have been used to quantify differences in temperature among various body parts (Sannolo et al. 2014), or to assess to what extent lizards can select the best spots for thermoregulation (Goller et al. 2014). Still, it is challenging to compare the new data collected from IR cameras with those obtained from thermocouples. Indeed, IR cameras only record surface temperature for most objects, including lizards (gases deserve a whole separate discussion; see chapter 8 in Vollmer and Möllmann 2018), while thermocouples are usually inserted into the cloaca and, hence, measure the internal temperature. Depending on body size (especially on the diameter), temperature gradients often exist between the core and surface temperatures of an animal (Gagge and Gonzalez 1996), not to mention that readings from different tools may differ only due to calibration.

The first significant result of this thesis has been to demonstrate that surface, and core temperature in different species of lizards correlate well, both in a big lacertid lizard like *Timon lepidus*, as well as in a small lizard like *Podarcis guadarramae*. The results exposed in the article I demonstrated that skin measurements obtained with an IR camera could be used to predict core temperatures, at least in a controlled environment. Secondly, the temperature gradient existing from the skin surface to the core of the body cherish the debate about which body temperature lizards are actually sensing and trying to regulate. Indeed, while thermic sensors are widespread in the body (Jordt et al. 2003), the control center of temperature is in the brain (Ralph et al. 1979; Morrison and Nakamura 2011). However, skin temperature is often warmer than core temperature under intense radiation, while the reverse is generally true under cooling (Christian et al. 2006). Thus, infrared data can contribute to understanding how the physical environment interplays with an individual’s physiology to determine its body temperature.

If data collected with IR cameras are reliable and represent well the internal temperature of a lizard, it means that such tools can be used in the field to measure lizards’ body temperature from afar, without the need of capturing the animal, if not necessary. Hence, the second important result of this thesis is of ecological and behavioral relevance. Despite the vast knowledge accumulated in recent decades on hiding behavior (summarized in Cooper and Blumstein 2015), a few important aspects remain understudied. For example, many ectotherms are likely to suffer from a reduction in body temperature while hiding from a predator. They may be then forced to spend some time to regain heat and lose the opportunity to feed or mate. However, no work so far measured to what extent the body temperature of a lizard may decrease as a consequence of hiding from a predator. Again, it may seem a trivial problem only until you stop thinking about how to measure the body temperature of a lizard both *before* and *after* a predation attempt while trying to minimize your influence on the animal’s behavior. Indeed, to measure body temperature with thermocouples you need to capture and manipulate the animal, that once released will hardly behave naturally. The studies that tried to put thermal costs into the equation of hiding behavior never measured body temperature before and after hiding (Cooper and Wilson 2008; Martín and López 1999, 2010).

The results discussed in article II are the firsts in quantifying to what extent lizards body temperature decreases as a result of hiding from a predator. We also demonstrated that the thermal quality of the refuges plays an important role in modulating hiding behavior in lizards, a finding that has been already reported in the past (Cooper and Wilson 2008). Understand how lizards (or ectotherms in general) modulate their hiding behavior depending on the decrease of their body temperature and on factors like the thermal quality of a refuge is of paramount importance to gain basic knowledge on animal behavior, as well as to understand the ecological needs in terms of refuge requirements, and to develop more powerful and accurate models that predict hiding behavior (Cooper and Blumstein 2015).

Advancements in the industry have been often a prerequisite for improvements in thermal ecology studies, at least at the logistical level. Just as an example, the IR camera used in the present work was developed by FLIR©, a leading company in designing thermal imaging tools for military, commercial and markets purposes. Such advancements will be much needed also in the future. For example, commenting on article II about the IR camera that was used in the field, a reviewer pointed out that we needed a higher resolution to get detailed images of lizards from afar. He was right, of course. By the time this thesis will be outdated, more powerful (and affordable, which is the real issue) IR cameras will be available to push further the investigation on thermal ecology. For now, it can be concluded that IR cameras can provide invaluable data on reptile thermal ecology and open new opportunities to study the relationship between an individual and its surrounding environment, provided that IR tools are correctly used.

7.3 Interspecific differences in thermal ecology, water balance and performance in coexisting lizards

Closely related species that coexist attracted the attention of scientists due to the potential evolutionary consequences of competition (MacArthur and Levins 1964), character displacement (Brown and Wilson 1956), character release (Grant 1972) and heritability (Boag and Grant 1978). A classic example is represented by Darwin's finches, which have been extensively studied for over 40 years by Peter and Rosemary Grant (Grant and Grant 2006; Grant and Grant 2014).

Among reptiles, the best-studied system in terms of coexisting species, character displacement and adaptation is likely represented by the Caribbean *Anolis* lizards

(Schoener 1968). *Anolis* lizards are often classified in “ecomorphs” due to the strong coadaptation they show between morphology and habitat. For example, long-legged and heavy-bodied lizards walk less often and use larger perches than lighter-bodied species that walk more often and use narrower supports (Losos 1990). Such ecomorphs can evolve rapidly and repeatedly if lizards are exposed to novel environments (Losos et al. 1997).

Studies on coexistance and character displacement are much less common for other groups of lizards. For example, lacertid lizards as a family count fewer species than *Anolis* as a genus (on why tropical groups are more diverse, see for instance Mittelbach et al. 2007), and do not show the extreme morphological and physiological degree of adaptations exhibited by *Anolis* lizards. Indeed, lacertid lizards are most commonly found on rocks, walls or the ground, while arboreality is only occasional. As a result, while various aspects of lacertid lizard biology have been extensively studied, like phylogeny (Arnold et al. 2007; Pyron et al. 2013), distribution (Sindaco and Jeremčenko 2008), morphology (Vanhooydonck and Van Damme 1999), physiology (Bauwens et al. 1995; Castilla et al. 1999) and ecology (Arnold 1987; Salvador 2014), relatively less effort has been devoted in comparing the biology of coexisting species.

Among lacertid species, only a few both share a recent common ancestry, overlap over most of their distribution range and appear, at least superficially, morphologically similar enough to investigate the potential for competition and character displacement. In recent years, several studies focused on the *Podarcis* species living in the Iberian Peninsula, more specifically those belonging to the *Podarcis hispanicus* complex (Harris and Sá-Sousa 2002). In recent years several forms have been recognized as distinctive enough to be described as valid species (Pinho et al. 2008). However, most of them show a parapatric distribution, with little overlap in their ranges (Kaliontzopoulou et al. 2011a). Instead, a few species like *P. bocagei* and *P. guardarramae*, overlap over most of their distribution and rapidly became a research model, primarily to study patterns of morphological variation (Kaliontzopoulou et al. 2011b; Caeiro-Dias et al. 2018).

Here the attention has been instead focused on the potential ecophysiological differences between *P. bocagei* and *P. guardarramae*. Indeed, to what extent physiology is plastic among closely related species is still debated. For instance, thermal preferences have been viewed as potentially labile or rigid character depending on the case (Hertz et al. 1983). For coexisting lacertid lizards, little information is available. For example, two species belonging to different genera showed variation in their resistance to water loss (García-Muñoz and Carretero 2013).

The first investigation, exposed as article III, showed that, while thermal preferences differ only a little, resistance to water loss varies to a greater degree in these species.

Hence, we found support for rigidity in thermal choices and reported one of the first cases of interspecific variability in resistance to water loss for closely related species that coexist. Differential resistance in water loss may determine in the field variable patterns of activity and even restrains lizards' activity, depending on the environmental conditions. Thus, while lizards from different species may prefer similar temperatures under standardized conditions, they may conversely be sensitive to various combinations of temperature, humidity and evapotranspiration, that can eventually translate into interspecific differences in microhabitat use and daily and seasonal time of activity. A more comprehensive research framework that takes into account not only temperature but also humidity, vegetation cover and other physical aspects is rarely applied, even though the underlying principles were analysed in depth many years ago (Porter and Gates 1969; Gates 1980; Gaiger 2003). In recent years, a few attempts have been made to couple temperature and humidity or water availability in snakes and lizards (Dupoué et al. 2015; Dupoué et al. 2017).

A second study reported as article IV, focused instead on the whole-organism performance, investigating the running abilities of these two species. In this case, we found no difference between species, an overlap of preferred temperature with performance, and a broad insensitiveness of running performance with varying body temperatures. Hence, whole-organism performance appears conservative, regardless of the morphological differences between the two species. More interestingly, the fact that performance was maximized over a broad range of body temperature fits well with the ecology of the species, which are considered generalists, and their distribution range falling within a temperate region. A central question, familiar to most studies investigating whole-organism performance, remains: to what extent lizards use their full running potential under natural conditions? The few data available indicate that, in general, lizards do not use their full potential (Hertz et al. 1988), but occasionally they do, especially during the mating season and in the context of intraspecific interactions (Braña 2003).

The results presented in chapter 5 all came from laboratory experiments. Indeed, while field temperatures are easy to obtain and compare among sexes, populations or species, it is challenging to collect and compare data on water balance. Some recent studies are comparing plasma density as a measure of dehydration (Dupoué et al. 2017). While this protocol allows for standardized comparisons among groups, its main drawback lies in lacking an absolute scale of reference. The lack of a dehydration reference scale is an issue that will likely limit our comprehension of water balance in the near future. Hence, it remains mostly unknown to what extent closely related species may differ in nature. Indeed, the *Podarcis* species studied here can be found in nature even on the same rock. Hence, field data on field body temperature, potential overlap in time of activity, and behavior are much needed to understand if, and to what extent, these species can coexist showing relatively little character displacement.

7.4 Resistance to water loss and dehydration in reptiles

Reptile thermal ecology has been intensively studied for more than a century now, and an impressive amount of data has been collected so far. Indeed, a query on Google Scholar for “Thermal ecology reptile” would return more than 27.000 results. From the first observations that, despite the common belief, reptile’s blood was not “cold” and that reptiles were able to behaviorally regulate their body temperature (Cowles and Bogert 1944; Brattstrom 1965), studies on thermal ecology of reptiles are nowadays contributing to the growing evidence that humans are causing the sixth mass extinction on Earth (Sinervo et al. 2010; Barnosky et al. 2011).

Instead, other aspects of reptile biology have been almost neglected. As Albert Szent-Györgyi wrote in 1971, “biology has forgotten water, or never discovered it”. This statement is still valid today for land reptiles. For historical reasons (i.e. Linnaeus), amphibians and reptiles have been studied together by researchers called “herpetologists” for decades. While for most amphibian species the need for a source of water was evident, reptiles seemed the perfect example of the adaptation to dry climates. Reptiles evolved the amniotic egg, which freed them from the need of laying eggs in an aquatic environment. In adults, keratinized integument reduces water loss through the skin, and their waste products are insoluble. Reptilian epidermis represent a major adaptation to land-dwelling life style (Alibardi 2003).

As Stephen Jay Gould once commented in an interview (Gould 1996), horses’ evolution is often quoted as a classic example of the spectacular forms created by natural selection. However, with a sudden steer, Gould explained that odd-toed ungulates (order Perissodactyla) are nowadays just a relict, a failed experiment. Most of the species of the order went gone extinct during the last 50 my, and nowadays only 17 species still survive. Not sure to what extent the metaphor may hold, I believe that land reptiles have often been quoted as an example of resistance to dry environments while many species are much more sensitive to water loss than previously thought.

Even though reptilian thermoregulation and water balance have often been discussed in neighboring chapters, and still are (Gans 1982; Pough et al. 2004), generally little crossing between the two occurs. Indeed, as far as I know, the first study that demonstrated that dehydrated lizards are forced to reduce their activity and thermoregulate to lower temperatures was published only 32 years ago (Crowley 1987). Soon after, other researchers reported the adverse effects of dehydration also on whole-organism performance (Wilson and Havel 1989). More studies investigated this issue in the following years, and research showed that dehydration, or water shortage, may reduce growth rate (Lorenzon et al. 1999) and modulate activity in lizards (Ryan et al. 2016). Article V contributed to demonstrating that dehydrated lizards thermoregulate less efficiently, and often retreat inside refuges to reduce further loss of water. Article VI showed that while field body temperatures are flexible and can change seasonally, resistance to water loss is more rigid. The evidence collected in Article V and IV may have profound ecological consequences. The most important repercussion of a trade-off between the need of thermoregulation and the necessity of reducing water loss is that lizard may be forced into situations in which the only potential choice may be to hide and wait for more favourable conditions. This contingency has been studied in desert lizards forced to interrupt activity due to extremely high diurnal temperatures and has been termed "hours of restriction in activity" (Sinervo et al. 2010). While lizards living in temperate regions are not be exposed to temperatures as high as those experienced in the desert, they may face a shortage of water availability, especially during the potentially prolonged summer droughts characteristic of Mediterranean climates. Several authors reported that lizards in summer appear to be less active, and often patterns of activity have been defined as bi-modal, with higher peaks of activity in spring and fall. The results of Article V and VI contributed significantly to the issue, suggesting that water balance and dehydration may help in explaining the pattern of reduced activity under conditions of high temperature and low water availability.

I believe that results like those presented in article V and VI will increasingly gain value during the next years. Indeed, while much research effort is devoted every day in understanding the potential effect that the global increment in mean air temperature may have on biodiversity (IPCC 2014), much less attention is dedicated to other aspects, like water availability. While almost half of humanity does not have today access to either safe drinking water or sanitation systems (Gleick 1988), the situation is likely to worsen in the future (Vörösmarty et al. 2000). For example, the increase in mean air temperature will likely lead to a reduction in water availability in cold climates as well as in tropical ones (Barnett et al. 2005; IPCC 2014), and droughts will become more frequent (Dai 2013). To aggravate the matter and make forecasting more difficult, the rate of climate change and the frequency of extreme events like heatwaves are not globally uniform (Urban 2015). For instance, Europe is experiencing higher rates of warming than most of the world (Walther et al. 2002), and southern European countries are those facing the worse projections (Schär et al. 2004) Unfortunately, the potential consequences of increasing temperature and lack of water availability are still poorly studied in animal populations. In birds, for example, the increase in the frequency of heatwaves may lead to catastrophic mortality, due to the rise of unsustainable rates of water loss (McKechnie and Wolf 2009). In reptiles, local extinctions have been reported in populations living in the desert or arid areas, likely due to the restriction on activity imposed by extremely high temperatures (Sinervo et al. 2010).

For European reptiles, data are scarce and invariably focused only on temperature. A recent study claims that some populations of Iberian lizards may face up to over 600 hours of restriction in activity per year (Herrando-Pérez et al. 2018). Even though this study used meteorological data at a too coarse scale (25 km^2) to be experienced by lizards (Geiger et al. 2003), the results are still worrisome. Furthermore, climate change is likely to increase the rate of desertification in many areas, as a result of the interplay between increasing air temperature, evapo-transpiration and changes in land-use (Le Houérou 1996; Puigdefábregas and Mendizabal 1998). Thus, reptile species, like lizards, will not only face the increase in average temperatures and the alteration of rainfall regimes, but also habitat degradation and destruction. The phylogenetic signal in resistance to water loss may determine divergent responses depending on the specific taxon.

The data presented in this work highlight how various species of lizards may be sensible to dehydration, and that different populations may react differently to water loss. High temperatures and dehydration may interact and contribute to reducing lizard activity, which in turn may negatively affect populations viability and survival. Clearly, we need more research to understand how European lizards may react to the current and future environmental challenges the face.

7.5 Future directions

The effort in studying thermal ecology, especially of ectotherms, is likely going to increase during the next decades. While during the first years of research on the subject the focus was mainly on understanding if and how reptiles thermoregulate, during the following decades many studies focused on developing accurate biophysical modeling of lizard thermoregulation and the thermal relations between an ectotherm and its environment. In more recent years, thanks to diffusion of both powerful computer, software and databases, it increases exponentially the interest in modeling the past present and future species distributions. During the last decade, the attention has shifted to study the potential effect that the global climate change may have on reptile biodiversity, distribution, and life-history. However, models are just tools, not the goal, and they need robust and reliable data to be both developed and tested.

In this context, studies investigating the field thermal ecology of reptiles will be much needed in the next years. What I mainly find missing in the literature is long-time series data of thermal ecology. Most studies, like the one presented here, focus only on a season. But if we want to assess the potential impact of a warming world on ectotherms, we need baselines and long-term data. Resident scientists —in particular— should devote more time in monitoring the thermal ecology of various population along several years. A recent line of investigation is linking thermal ecology, water balance and morphology. While reptiles have been traditionally viewed as virtually unaffected by aridity, many species living in temperate regions appear much more sensitive to water availability than previously thought. Similarly, several studies —especially during the ’80 of the previous century— investigated the route of water loss through the skin (mainly in snakes), concluding that dermal lipid composition and thickness was the barrier for cutaneous water loss. Still, recent investigations are pointing out the potential role of scale morphology and the degree of skin stretching in modulating cutaneous water loss in lizards. A deeper understanding of the routes of water intake and of the morphology and physiology of water loss in lizards will be a much-needed complement to ecological and modeling studies if we want to understand how reptiles are facing the challenges of the Anthropocene.

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Chapter 8: Appendices

“The chase is better than the catch.”

— Anonymous

Appendix A: Supplementary Material of Article I

Supplementary Materials for:

Assessing the reliability of thermography to infer internal body temperatures of lizards

Frederico M. Barroso [a,b], Miguel A. Carretero [a], Francisco Silva [c] , Marco Sannolo [a,c,*]

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In the present Supplementary Materials we present relevant Post-hoc tests carried out on the Linear Mixed Effect Models. The starting formula was:

*Body temperature ~ Treatment*Body Position*Species*

The model was reduced accordingly to Zuur et al. (2009), until only significant factor or their interactions remain. After reduction, the following all two-ways interactions were significant (see section 3.3). Here we publish the results of Post-hoc analysis run with the lmerTest package on the significant interaction terms.

*Treatment*Species*

Regardless of the species, the two treatments (cooling versus heating) imposed different body temperatures on all species (P < 0.0001). Similarly, each species differed in body temperature depending on the treatment (P < 0.0001). However, the pairwise comparison of the three species not always showed significant differences in temperature within treatment (see Table S1).

Treatment: Heating		
P-value		
P. virescens	*L. schreiberi*	**< 0.0001**
P. virescens	*T. lepidus*	> 0.05
L. schreiberi	*T. lepidus*	> 0.05
Treatment: Cooling		
P. virescens	*L. schreiberi*	**< 0.0001**
P. virescens	*T. lepidus*	**< 0.0001**
L. schreiberi	*T. lepidus*	> 0.05

Table S1. Pairwise comparisons of body temperature of the three species for each treatment.

*Treatment*Body Position*

In Table S2 we present the results for the effect of treatment and body position on the body temperature.

Heating							**Cooling**						
Podarcis virescens													
Body position	**P-value**						**Body position**	**P-value**					
Snout	-	-	-	-	-	-	Snout	-	-	-	-	-	-
Eye	>0.05	-	-	-	-	-	Eye	>0.05	-	-	-	-	-
Head	>0.05	>0.05	-	-	-	-	Head	>0.05	>0.05	-	-	-	-
Dorsum	**=0.029**	**=0.028**	>0.05	-	-	-	Dorsum	>0.05	>0.05	>0.05	-	-	-
Leg	>0.05	>0.05	>0.05	>0.05	-	-	Leg	>0.05	>0.05	>0.05	>0.05	-	-
Tail	>0.05	>0.05	>0.05	>0.05	>0.05	-	Tail	>0.05	>0.05	>0.05	>0.05	>0.05	-
Cloaca	>0.05	>0.05	**<0.001**	**<0.001**	>0.05	**=0.001**	Cloaca	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Lacerta schreiberi													
Snout	-	-	-	-	-	-	Snout	-	-	-	-	-	-
Eye	>0.05	-	-	-	-	-	Eye	>0.05	-	-	-	-	-
Head	>0.05	**=0.001**	-	-	-	-	Head	>0.05	>0.05	-	-	-	-
Dorsum	>0.05	**<0.001**	>0.05	-	-	-	Dorsum	>0.05	>0.05	>0.05	-	-	-
Leg	>0.05	**=0.005**	>0.05	>0.05	-	-	Leg	>0.05	>0.05	>0.05	>0.05	-	-
Tail	>0.05	**<0.001**	>0.05	>0.05	>0.05	-	Tail	>0.05	>0.05	>0.05	>0.05	>0.05	-
Cloaca	**=0.024**	>0.05	**<0.001**	**<0.001**	**<0.001**	**<0.001**	Cloaca	>0.05	>0.05	**=0.006**	**=0.002**	**=0.022**	**=0.003**
Timon lepidus													

Snout	-	-	-	-	-	-	Snout	-	-	-	-	-	-
Eye	>0.05	-	-	-	-	-	Eye	>0.05	-	-	-	-	-
Head	>0.05	**<0.001**	-	-	-	-	Head	>0.05	>0.05	-	-	-	-
Dorsum	>0.05	**<0.001**	>0.05	-	-	-	Dorsum	>0.05	**=0.036**	>0.05	-	-	-
Leg	>0.05	**=0.008**	>0.05	>0.05	-	-	Leg	>0.05	>0.05	>0.05	>0.05	-	-
Tail	>0.05	**<0.001**	>0.05	>0.05	>0.05	-	Tail	>0.05	>0.05	>0.05	>0.05	>0.05	-
Cloaca	>0.05	>0.05	**<0.001**	**<0.001**	**<0.001**	**<0.001**	Cloaca	>0.05	>0.05	=0.036	**=0.01**	>0.05	**=0.026**

Table S2. Effect of of treatment and body position on the body temperature.

Species*Body Position

In Table S3 we present the comparison of each body position for the three species, regardless of the treatment (i.e. averaging cooling and heating)

Species comparison	**Body position**						
	Snout	Eye	Head	Dorsum	Leg	Tail	Cloaca
P. virescens vs. *L. schreiberi*	**<0.0001**	**=0.009**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	>0.05
P. virescens vs. *T. lepidus*	**=0.007**	>0.05	**=0.007**	>0.05	**=0.003**	**=0.003**	>0.05
L. schreiberi vs. *T. lepidus*	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Table S3. Comparison of body temperature for each body part for the three species.

Appendix B: Supplementary Material of Article II

Supplementary material of:

Waitin' on a sunny day: lizards body temperature decreases while hiding from predators

Marco Sannolo, Raquel Ponti, Miguel A. Carretero

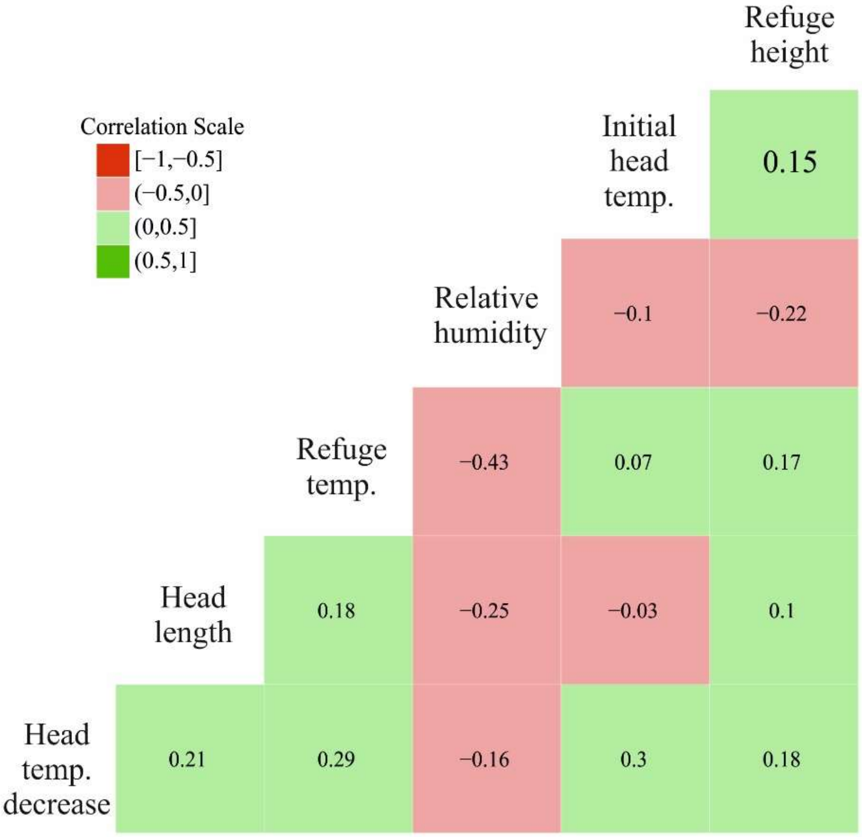
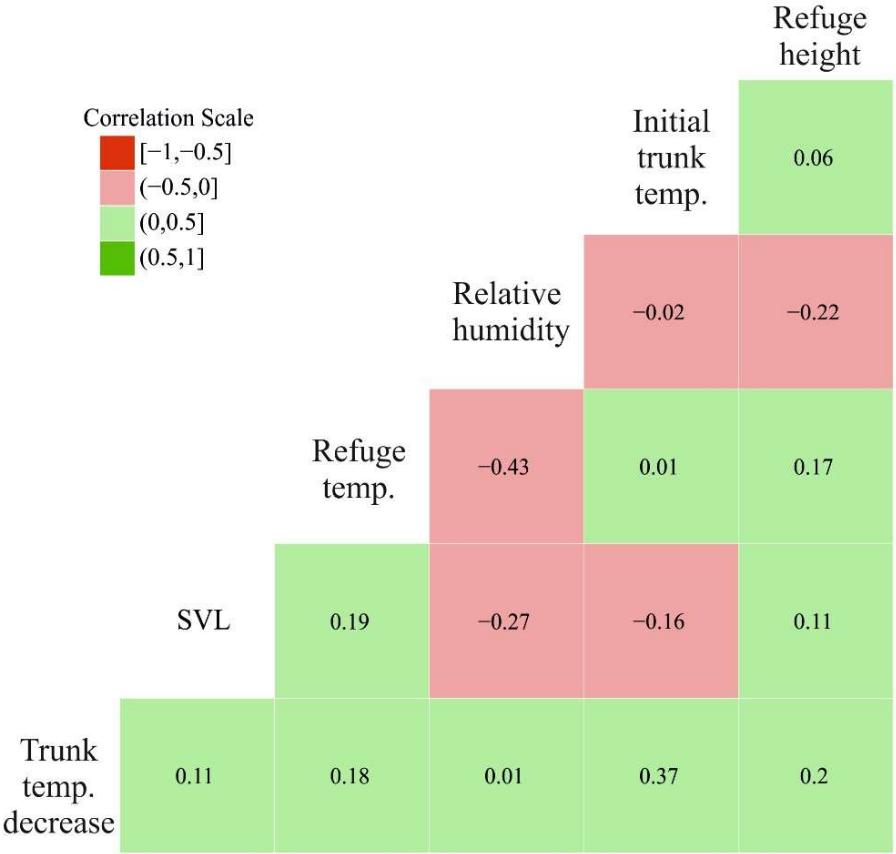


Fig. S1. Pairwise plot of correlations for the predictors used in SEM analysis of appearance time.



	SVL	Refuge temp.	Relative humidity	Initial trunk temp.	Refuge height
Trunk temp. decrease	0.11	0.18	0.01	0.37	0.2
SVL		0.19	−0.27	−0.16	0.11
Refuge temp.			−0.43	0.01	0.17
Relative humidity				−0.02	−0.22
Initial trunk temp.					0.06

Fig. S2. Pairwise plot of correlations for the predictors used in SEM analysis of recovery time.

Table S4. VIF values of the predictors used SEM analysis of appearance time.

Head temperature decrease	1.253175
Refuge temperature	1.313871
Refuge humidity	1.315062
Initial head temperature	1.132850
Head length	1.118801
Refuge height	1.091254

Table S5. VIF values of the predictors used SEM analysis of recovery time.

Trunk temperature decrease	1.309742
Refuge temperature	1.292482
Refuge humidity	1.369485

Initial trunk temperature	1.237788
SVL	1.163119
Refuge height	1.104157

Appendix C: Supplementary Material of Article III

Physiological differences in preferred temperatures and evaporative water loss rates in two sympatric lacertid species

Supplementary Materials

Here we present additional figures, complementary to those presented in the main text.

Table S6. Individuals that exceeded 5% of water loss during the experiment, divided by species, temperature and time of occurrence.

	27°C		**32°C**		**37°C**	
Time	*P. bocagei*	*P. g. guadarramae*	*P. bocagei*	*P. g. guadarramae*	*P. bocagei*	*P. g. guadarramae*
5			2	1	3	3
6					1	
7	1			2	1	3
8		2	1	1	2	1
9	2	1	1	3	2	3

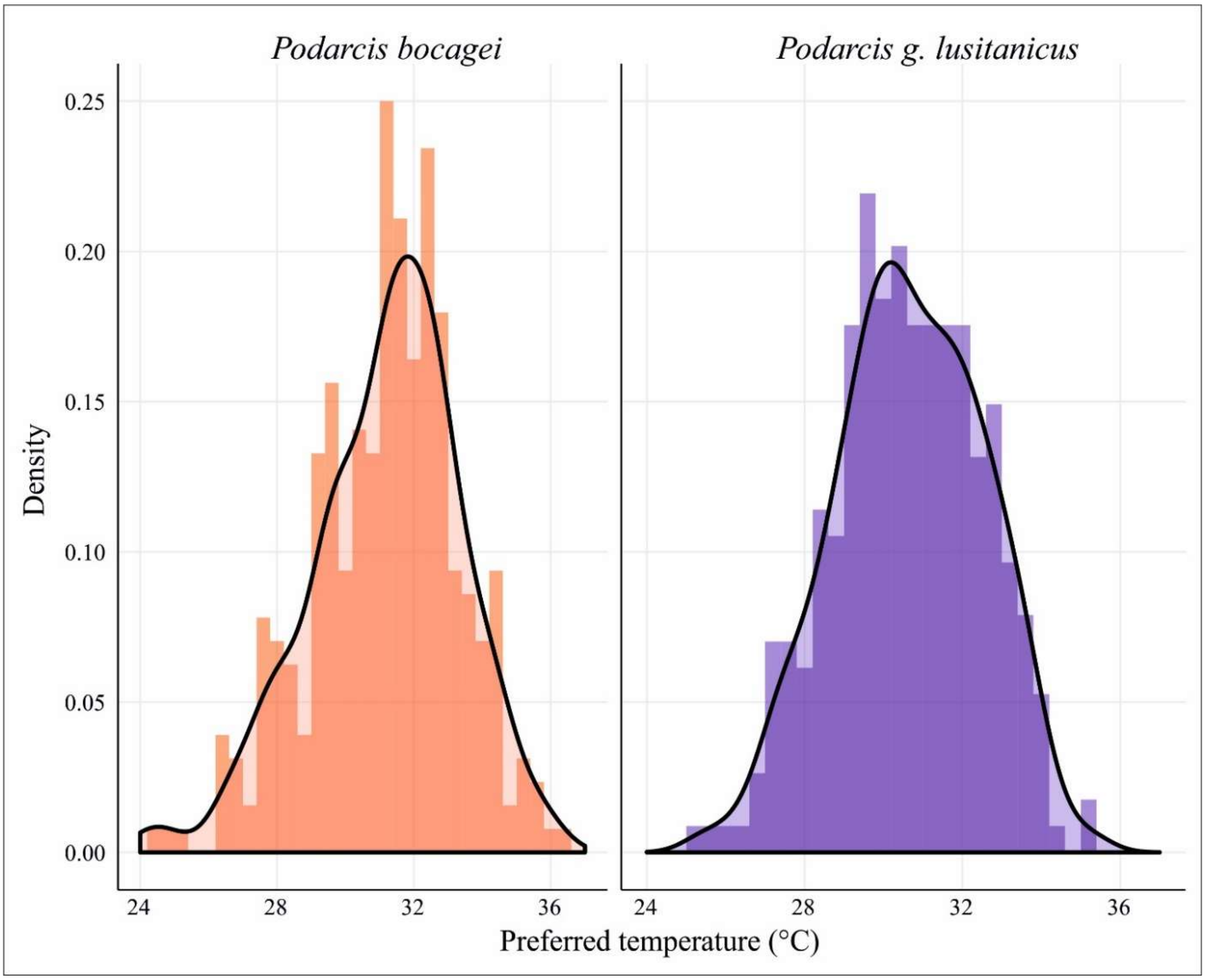


Fig. S3. Distribution as density histogram of preferred temperature for *Podarcis bocagei* and *P. g. lusitanicus*.

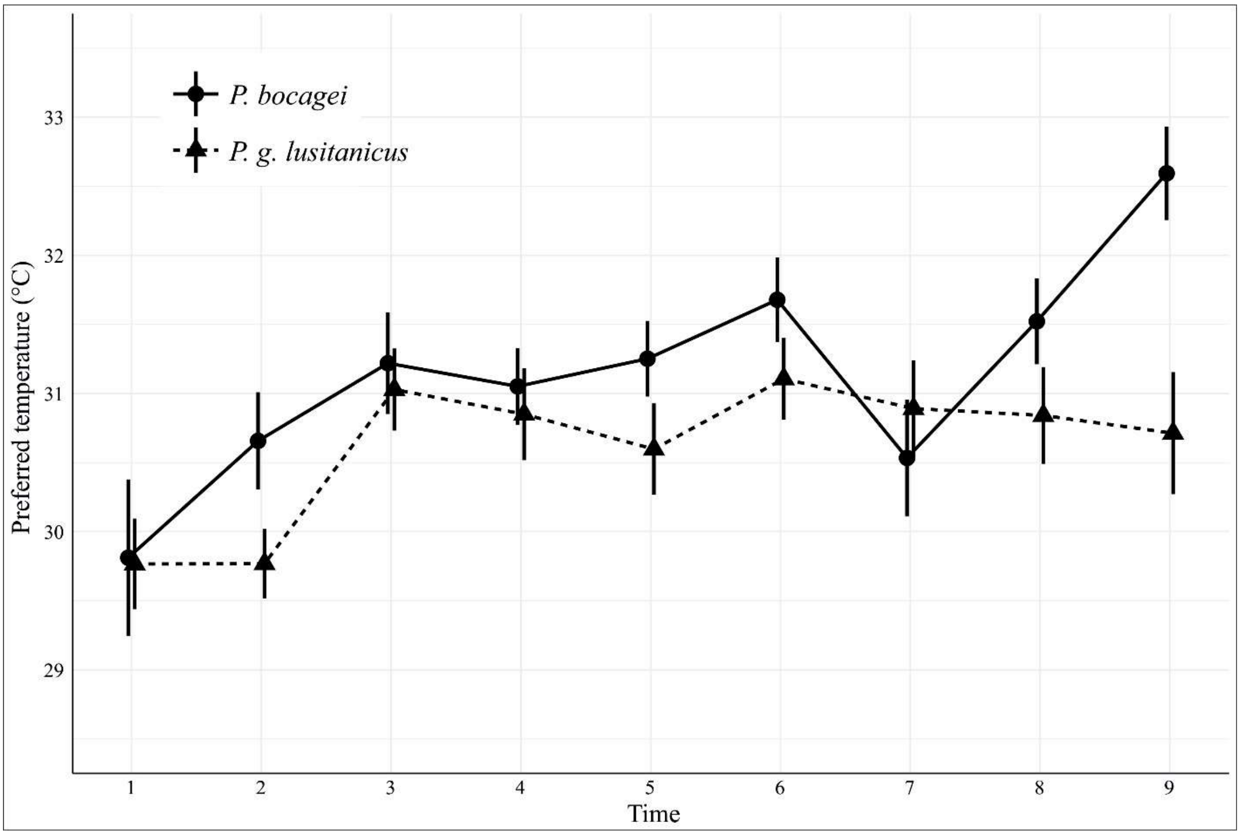


Fig. S4. Preferred body temperature for both lizard species over time.

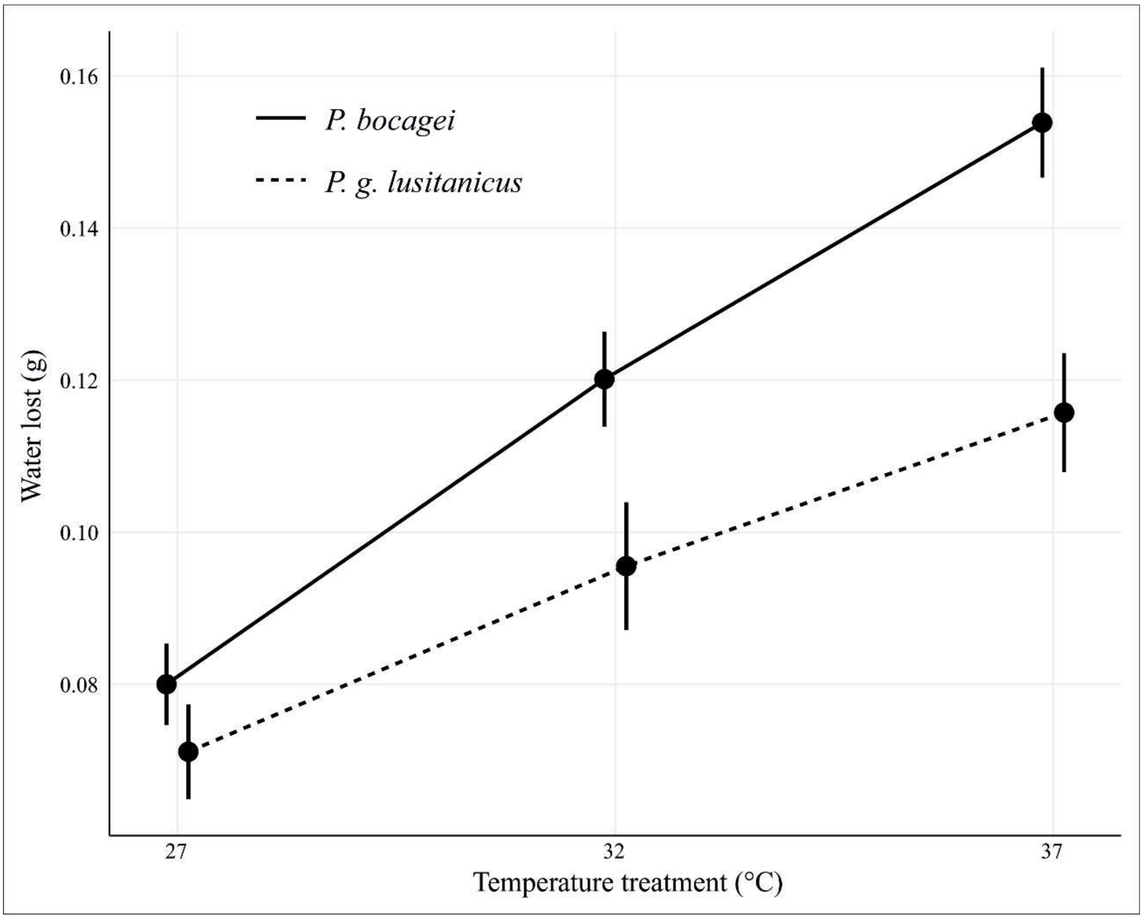


Fig. S5. Water loss rate as absolute values for *P. bocagei* and *P. g. lusitanicus* at all temperature treatments.

Appendix D: Supplementary Material of Article V

Appendix

Skewness

The significance of skewness in body temperature was calculated using the D'Agostino test with the *moments* library in R. The *p.adjust* function was used to account for individual repeated measures (using the correction of Benjamini and Yekutieli, 2010).

Species	Treatment	Skewness	Z-value	*P* original	*P* adjusted
P. bocagei	Water	-0.79	-5.053	2.176×10^{-07}	6.373×10^{-06}
	No Water	-0.78	-4.967	2.176×10^{-07}	2.176×10^{-07}
P. carbonelli	Water	-0.61	-3.791	7.507×10^{-05}	0.0022
	No Water	-1.12	-6.146	3.974×10^{-10}	1.163×10^{-08}
P. guadarramae	Water	-0.52	-3.580	0.0002	0.0050
	No Water	-0.90	-5.694	6.219×10^{-09}	1.822×10^{-07}
P. virescens	Water	-0.82	-5.423	2.929×10^{-08}	8.579×10^{-07}
	No Water	-1.42	-8.140	2.22×10^{-16}	6.502×10^{-15}

Table S7. Skewness in preferred body temperature in four species of *Podarcis* lizards subjected to two treatments. In "water" treatment, individuals had had full access to a water source throughout the experiment. On the contrary, a water source was not available during the "no water" treatment. Skewness was calculated for both treatment, and its statistical significance tested with D'Agostino test. Original P-values are reported as well as corrected ones for multiple individual measurements (for each lizard body temperature was measured ten times).

Interspecific differences in reaction to dehydration

Even though some of the tested species differ in their thermal preference when fully hydrated, their body temperature did not differ when dehydrated. Such a result suggests that, despite having different thermal ecology under optimal conditions, dehydration likely impose similar physiological constrains on all tested species, forcing them to converge on comparable, lower body temperatures.

Pair comparison	Mean difference (°C)	*t*-value	*P*
P. bocagei – P. carbonelli	-0.37	-1.207	0.4548
P. bocagei – P. guadarramae	**-0.80**	-3.530	**0.0021**
P. bocagei – P. virescens	0.00	0.520	0.6033
P. carbonelli – P. guadarramae	-0.43	-2.177	0.1176
P. carbonelli – P. virescens	0.37	1.733	0.249
P. guadarramae – P. virescens	**0.80**	4.141	**0.0002**

Table S8. Mean values from which differences are calculated are shown in Table S6. Significance of pairwise comparison was calculated after fitting for the general model as explained in the method section. Bold values represent statistically significant comparisons.

Environmental temperature in the gradients

At the end of the experiment, we randomly chose two gradients. In each, we placed six iButton Thermochron® (Model DS1923; Maxim Integrated Products, Sunnyvale, CA, USA). Each datalogger was positioned in the middle of each of the five sections into which the gradients were virtually divided. The sixth datalogger was placed inside the refuge. Each datalogger was 20 cm away from the next, except the first one, that was close (~ 7 cm) to the one positioned inside the refuge.

Dataloggers were set to record temperature every 30 mins and were retrieved after seven days. We merged the data from the two gradients and of the six positions to create a distribution of temperature for each section of the gradients.

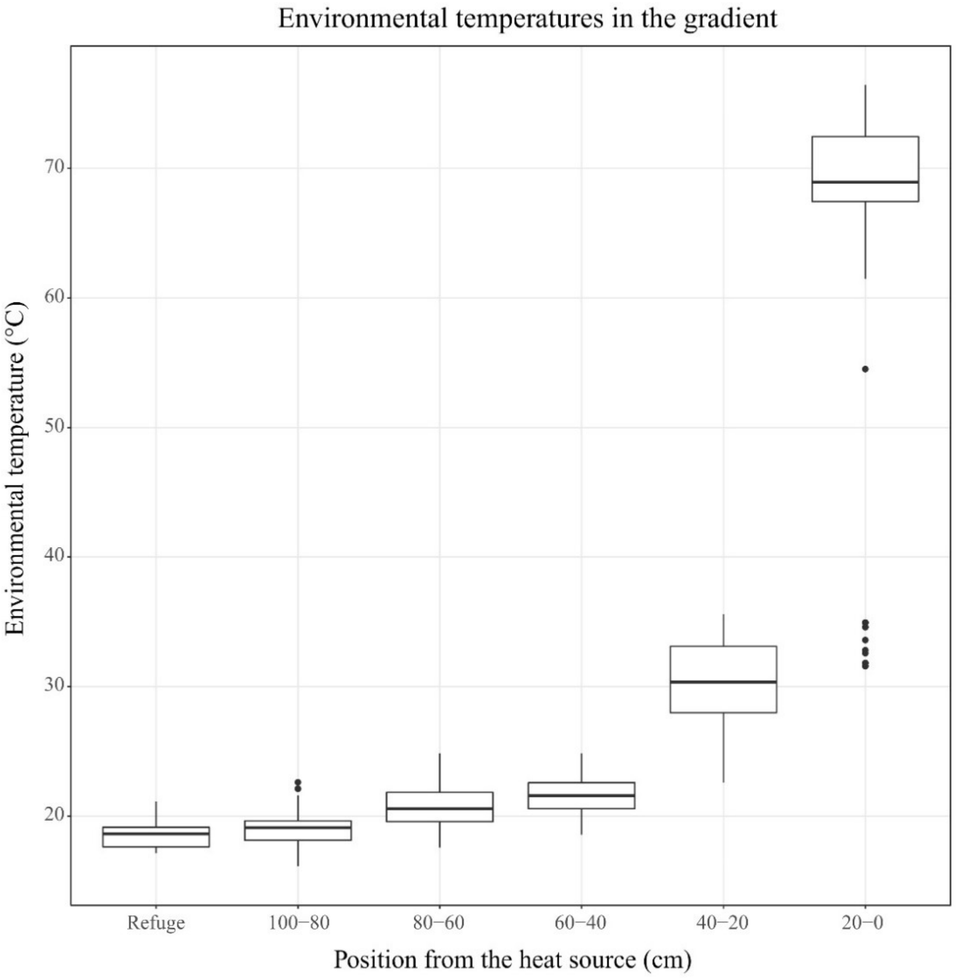


Fig. S6. Boxplot of environmental temperatures available in the gradients as measured with dataloggers.

Position (from the heat	n	Mean ± SD
0 - 20	294	68.03 ± 8.35
20 - 40	294	30.27 ± 3.23
40 - 60	294	21.47 ± 1.23
60 - 80	294	20.63 ± 1.63
80 - 100	294	19.05 ± 1.26
Refuge	294	18.62 ± 0.95

Table S9. Mean environmental temperatures ± standard deviation for each section of the gradient.

Appendix E: Supplementary Material of Article VI

Supplementary Material of:

Variation in field body temperature and resistance to dehydration along an environmental gradient in a diurnal ectotherm

Marco Sannolo, Emilio Civantos, José Martín, Miguel Angel Carretero

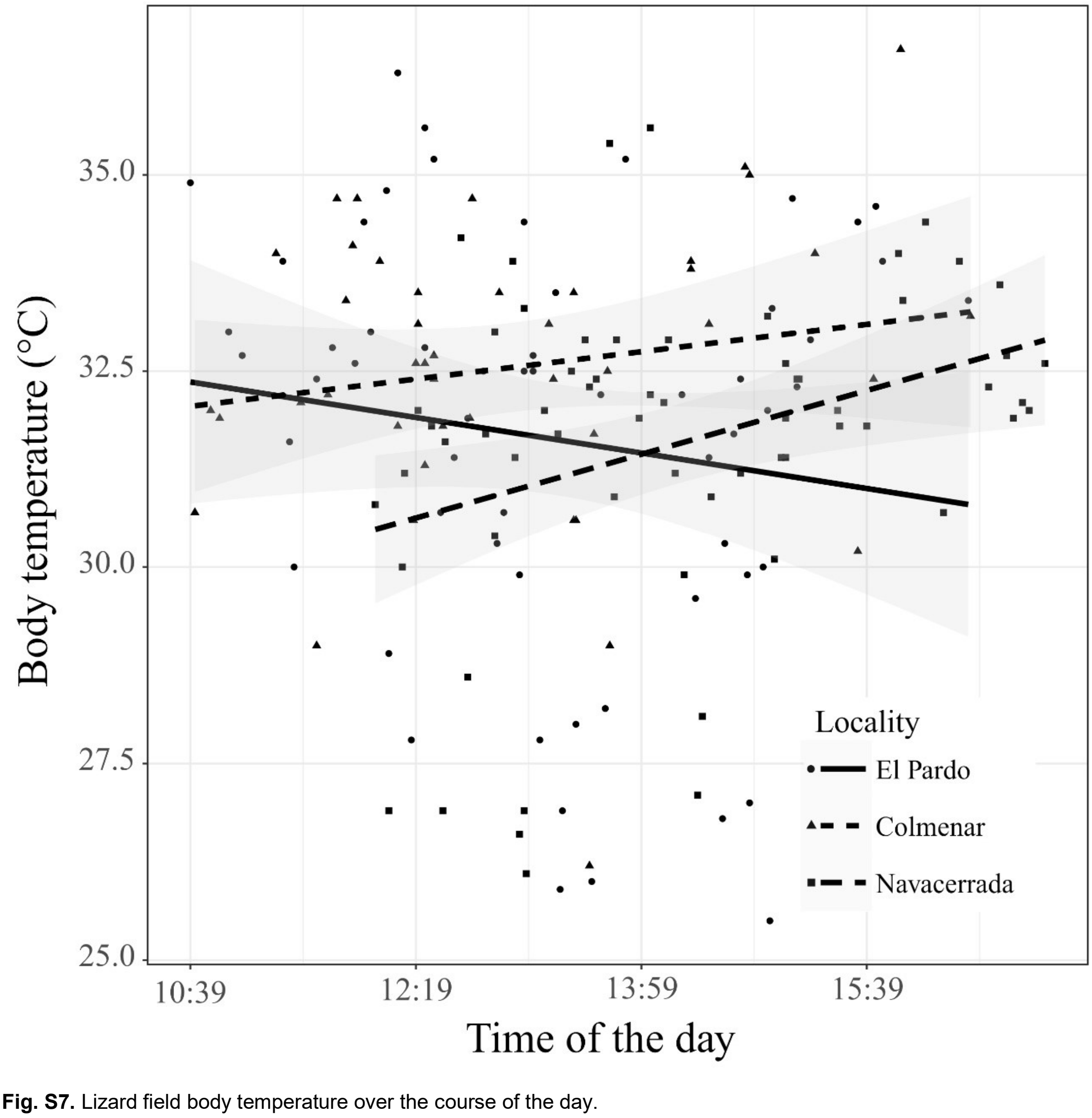


Fig. S7. Lizard field body temperature over the course of the day.

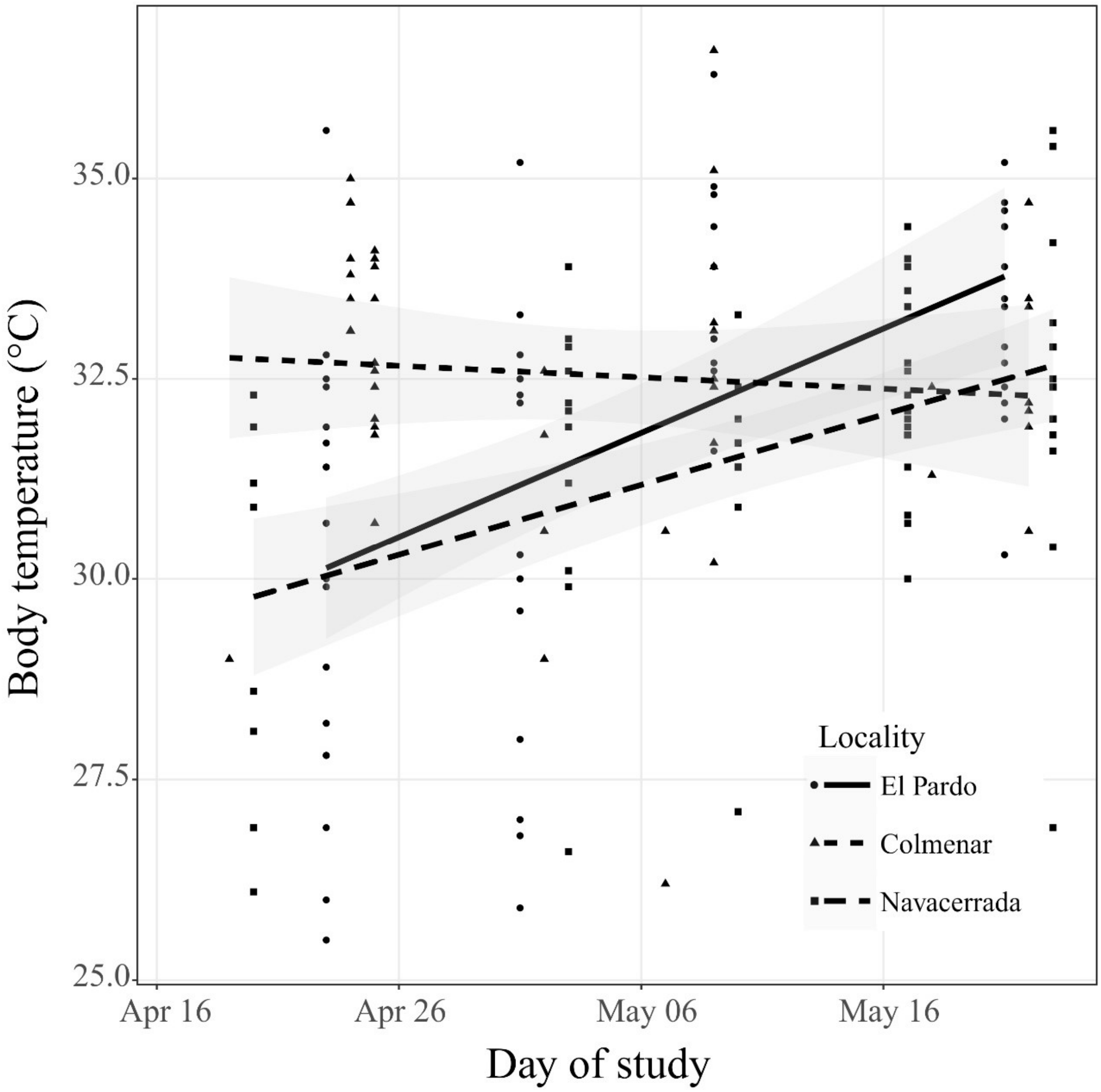


Fig. S8. Lizard field body temperature and day of sampling.

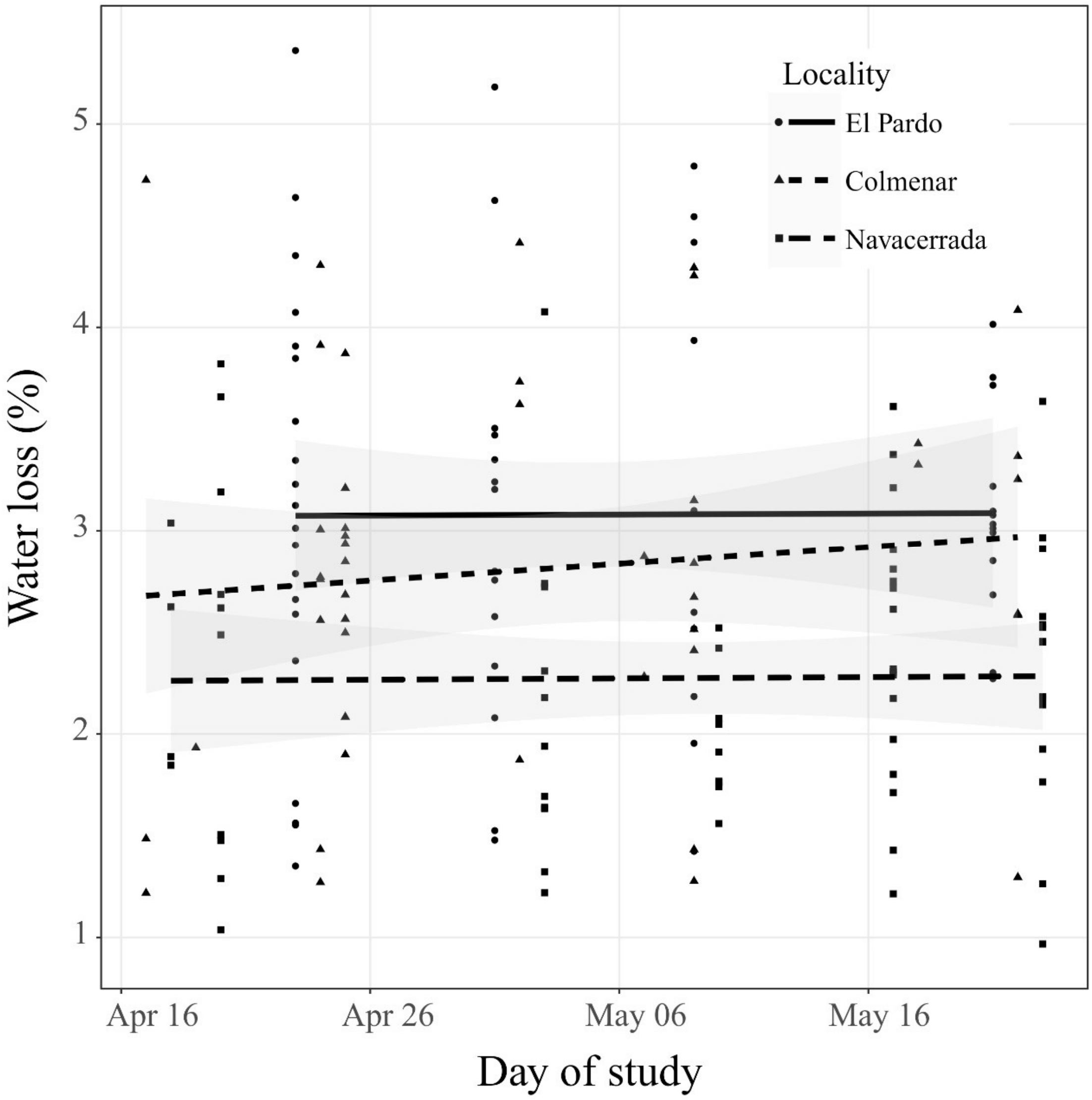


Fig. S9. Lizards’ resistance to water loss and day of sampling.

Appendix F: Other publications during the Doctoral program

The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2015, ••, ••–••. With 1 figure.

Does a polymorphic species have a 'polymorphic' diet? A case study from a lacertid lizard

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Lizards are ideal for studying colour polymorphism, because some species are polymorphic and the morphs often have different ecological or reproductive strategies. We studied the feeding habits of six polymorphic populations of *Podarcis muralis* to test whether morphs differed in their diet. Some taxa were selected in a similar way by all morphs, but selection on other taxa varied and was characteristic of each morph. Diet was most different for the red and yellow morphs. Two hypotheses could explain these differences: active segregation in the trophic niche or active segregation in space dependent on spatial heterogeneity in prey availability. The former is improbable because *P. muralis* is considered an opportunistic feeder, whereas the latter could occur if the morphs adopted alternative territorial strategies with consequent spatial segregation. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **00**, 000–000.

ADDITIONAL KEYWORDS: colour polymorphism – feeding habits – *Podarcis muralis* – trophic niche.

INTRODUCTION

Colour polymorphism, i.e. the coexistence in one interbreeding population of two or more distinct and genetically determined colour forms (Huxley, 1955), has attracted increasing interest over the past two decades, particularly with respect to the function of colours and ongoing selection on these traits in the wild (Gray & McKinnon, 2007; McKinnon & Pierotti, 2010; Olsson, Stuart-Fox & Ballen, 2013). Indeed, identifying the processes that lead to the evolution and maintenance of links between colour morphs and behavioural strategies is one of the most interesting topics in evolutionary biology (Gray & McKinnon, 2007), due to implications to the maintenance of genetic variation and the evolution of reproductive isolation and speciation processes (West-Eberhard, 1989; Pryke & Griffith, 2006; McKinnon & Pierotti, 2010). There is general agreement that the occurrence in the same population of different morphs reveals the occurrence of alternative ecological and reproductive strategies, which involve complex combinations of behavioural, morphological, physiological and life-history characteristics (Sinervo & Lively, 1996; Svensson, Sinervo & Comendant, 2001; Sacchi *et al.*, 2007a, 2009, 2015).

Lizards offer an ideal model to investigate the relationships between colour polymorphism and the ecological processes undergoing morph evolution and maintenance, as some lizard species show a significant colour polymorphism, such as *Urosaurus ornatus* (Thompson & Moore, 1991), *Uta stansburiana* (Sinervo & Lively, 1996), *Podarcis melisellensis* (Huyghe *et al.*, 2007), *Podarcis muralis* (Sacchi *et al.*, 2007b) and *Quedenfeldtia trachyblepharus* (Blouin-Demers *et al.*, 2013). Colour polymorphism

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ethology international journal of behavioural biology

Ethology

Common Wall Lizard Females (*Podarcis muralis*) do not Actively Choose Males Based on their Colour Morph

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Keywords: colour polymorphism, sexual selection, mate choice, chromatic and chemical signal

Abstract

Identifying the processes that lead to the evolution and maintenance of links between colour morphs and behavioural strategies has implications for the evolution of reproductive isolation and sympatric speciation. Sexual selection may play a significant role in the evolution of colour pattern complexity in reptiles, particularly when there are fitness consequences associated with mating with males of different colour morphs. In this article, we explored if common wall lizard females (*Podarcis muralis*) actively select males according to their morph in a colour-assortative pattern using a multiple-choice experiment with both visual and chemical cues. We failed to identify female active mate choice, as females did not choose males based on male colouration or femoral pore secretions. Indeed, females equally entered the three preference compartments and spent nearly the same amount of time within them, irrespective of both colour and odour of males. Consequently, our results do not support the hypothesis that colour polymorphism in this species may be driven by colour-assortative mating promoted by females. However, we cannot exclude the possibility that females may choose males according to their colour following a flexible choice strategy, nor the possibility that females actively discriminate among males according to qualities that are not directly related to morph-specific strategies.

Introduction

Identifying the proximal mechanisms leading to the evolution and maintenance of colour polymorphism has relevant consequences on the understanding of evolutionary processes, particularly the evolution of reproductive isolation and sympatric speciation (Huxley 1955; West-Eberhard 1986; Pryke & Griffith 2006; McKinnon & Pierotti 2010). Morphs are generally associated with the occurrence of alternative strategies, which involve complex combinations of behavioural, morphological, physiological and life history characteristics (Sinervo & Lively 1996; Svensson et al. 2001; Sacchi et al. 2007a, 2009). Therefore, colour morphs represent alternative, locally adapted optima (Calsbeek et al. 2010). Maintenance of alternative strategies within a single species is generally regarded as the result of the equilibrium between opposite selective pressures: disruptive selection (i.e., correlational selection), which promotes the association between life history traits and colouration, and the stabilizing selection (e.g., frequency dependent selection), which preserve gene flow among morphs (Sinervo & Svensson 2002; Noble et al. 2013). Because the equilibrium between alternative strategies is not necessarily stable under changing conditions, some morphs can be favoured whereas other may decline; thus peculiar phenotypes can be fixed through allelic selection thereby promoting sympatric speciation (i.e., exclusive expression of only one alternative, West-Eberhard 1986). Morph fixation may be accompanied by 'character release' and fast divergence from

5

Digital identification and analysis

Roberto Sacchi, Stefano Scali, Marco Mangiacotti,
Marco Sannolo, and Marco A.L. Zuffi

5.1 Introduction

The identification of individual animals in a population (usually achieved through marking) aids in acquiring accurate estimates of population size in ecological studies. In reptiles, marking usually involves scale clipping, branding, tattooing, subcutaneous elastomer injections, PIT tags, painting, and toe-clipping (see Chapter 4). However, marking techniques involve capturing and handling animals, which can alter survival probability or other life-history traits leading to serious violations of the assumptions of demographic models (Bloch and Irschick, 2004).

Digital identification (i.e. photographic identification) of individual animals is a non-intrusive way to obtain information on life-history traits that is based on distinctive individual morphological characteristics (such as patterns of ornamentation or morphology; Figure 5.1) that remain unchanged over time despite skin moulting. With respect to other techniques, digital identification has the advantage of being relatively inexpensive (entailing only a digital camera and computer), requiring only basic expertise to manage image analysis tools, and allowing large numbers of individuals to be re-identified. Typically, it employs catalogues of images that are matched against new images in order to assess if they are re-sightings of previously photographed individuals (i.e. 'recaptures') or still unknown individuals (i.e. 'captures'), which would then be included in the catalogue.

A major constraint involved in this process is the large amount of time required for image acquisition and comparison, which is likely to increase exponentially with increasing sample size. Indeed, for small catalogues (i.e. <50 individuals), image matching is still feasible by eye (Stenhouse, 1985). When catalogue items exceed 100 or so, however, the identification process becomes hugely time-consuming and misclassification errors occur frequently (Gamble et al., 2008; Sacchi et al., 2010; Bolger et al., 2012). The increasing use of photo-identification techniques coincided with advances in image analysis tools and pattern recognition algorithms, which have accelerated the image matching process. For this reason, photographic identification has become a popular technique in reptile capture–recapture studies

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ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

Comparison of photo-matching algorithms commonly used for photographic capture–recapture studies

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Abstract

Photographic capture–recapture is a valuable tool for obtaining demographic information on wildlife populations due to its noninvasive nature and cost-effectiveness. Recently, several computer-aided photo-matching algorithms have been developed to more efficiently match images of unique individuals in databases with thousands of images. However, the identification accuracy of these algorithms can severely bias estimates of vital rates and population size. Therefore, it is important to understand the performance and limitations of state-of-the-art photo-matching algorithms prior to implementation in capture–recapture studies involving possibly thousands of images. Here, we compared the performance of four photo-matching algorithms; Wild-ID, I3S Pattern+, APHIS, and AmphIdent using multiple amphibian databases of varying image quality. We measured the performance of each algorithm and evaluated the performance in relation to database size and the number of matching images in the database. We found that algorithm performance differed greatly by algorithm and image database, with recognition rates ranging from 100% to 22.6% when limiting the review to the 10 highest ranking images. We found that recognition rate degraded marginally with increased database size and could be improved considerably with a higher number of matching images in the database. In our study, the pixel-based algorithm of AmphIdent exhibited superior recognition rates compared to the other approaches. We recommend carefully evaluating algorithm performance prior to using it to match a complete database. By choosing a suitable matching algorithm, databases of sizes that are unfeasible to match "by eye" can be easily translated to accurate individual capture histories necessary for robust demographic estimates.

KEYWORDS
AmphIdent, APHIS, capture–recapture, I3S, photographic identification, Wild-ID

1 | INTRODUCTION

Understanding species population dynamics is an important step toward successful conservation. Capture–mark–recapture (CMR) and capture–recapture (CR) models have proven to be very useful for estimating population demography and for testing ecological hypotheses (Cormack, 1964; Jolly, 1965; Lebreton, Burnham, Clobert, & Anderson, 1992; Seber, 1965). CMR studies typically require invasive techniques

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Stable or plastic body shape? *Emys orbicularis* hatchlings-juveniles growth patterns under different ecological conditions

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Abstract. To test if habitat could shape shell features of freshwater turtles starting from birth, we tested hatchlings of *Emys orbicularis*, randomly chosen from four clutches of the same locality, under two treatments, stagnant (pond habitat) vs lotic (river habitat) water regimes. Due to mortality at the beginning of the experiment, we finally considered only two clutches. Turtle shells were characterized initially at hatching and then after one year of treatment by means of traditional and geometric morphometrics, and phenotypic trajectories analyses were conducted on both types of data. Carapace growth was isometric both in proportion and shape, while plastron showed an allometric pattern: length increased more than width, while the contour showed an expansion at the rear and a compression of the front. The hydrodynamic conditions during growth did not seem to affect the phenotypic trajectories of the shell proportions or the plastron. On the contrary, a significant effect was found on the phenotypic trajectory of the carapace: the size component (but not the angle one) was positively influenced by the "lentic" treatment, suggesting a different growth rate between the two environmental conditions.

Key words: *Emys orbicularis,* hatchlings, contrasted habitats, growth patterns, body shape, phenotypic variation.

Introduction

The bony and epidermal carapace of Chelonians represents a structure usually considered less phenotypically plastic than soft parts. However, research showed that habitat features and feeding habits play a significant role in modifying carapace shape in terrestrial and aquatic species (Claude et al. 2003). Mechanical implications in sexual dimorphism have also been found relevant using shape analyses (Vega & Stayton 2011). Finally, fecundity selection in females and sexual selection in males have been suggested as the main sources of phenotypic variation in freshwater turtles (Bonnet et al. 2010). To date, there is limited research background for the dynamics of the shell growth and shape of freshwater turtles, especially during their first year, a key period that likely determines the final shape of the turtle's shell, and on the habitat conditions that might influence this process.

The speed of running water is a critical feature of the habitat in which aquatic species live and it characterizes many aspects of their biology. A strong relationship between stream speed and organism morphology has been often underlined in a wide range of taxa: plants (Puijalon & Bornette 2004, Boller & Carrington 2006, Stewart 2008), invertebrates (Marchinko 2003, Holomuzki & Biggs 2006), and vertebrates (Pakkasmaa & Piironen 2001, McGuigan et al. 2003, Peres-Neto & Magnan 2004, Rivera 2008). In most of the cited cases, the variations of shape have been interpreted as of adaptive value: individuals living in fast running waters show morphological characteristics that markedly reduce the strength of resistance to the water, thus improving their body structure. However, these directional adaptations may negatively affect performances in other contexts, for instance in prey-predator interactions or during thermal exchanges (Polo-Cavia et al. 2009).

Many species of fresh water turtles show a body development apparently caused by diet, feeding and habitat (Claude et al. 2003). Recent studies have also suggested that hydrodynamics represents a primary factor influencing intraspecific variation of carapace size and shape (Lubcke & Wilson 2007, Rivera 2008). The friction forces acting on the carapace and plastron increase in intensity equal to the squared difference between the

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Research paper

Seasonal variations of plasma testosterone among colour-morph common wall lizards (*Podarcis muralis*)

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ABSTRACT

Sexual steroids influence reproductive behaviours and promote secondary sexual traits. In male lizards, increasing levels of testosterone (T) bolster conspicuous colouration, stimulate territoriality, and trigger antagonistic interactions among rivals. Moreover, in colour polymorphic species, reproductive strategy, aggressiveness and T levels can differ between morphs. Therefore, T level is considered as an important mechanism that regulates the expression of colour polymorphism and sexual behaviours of males. But in the polymorphic territorial wall lizard (*Podarcis muralis*), a lack of relationship between morphs and aggressiveness challenges the notion that T plays such a role. To examine this issue, we compared adult T levels among three colour morphs (white, yellow and red) through repeated sampling during the mating season. High T levels were observed at the onset of the mating season followed by a significant decrease, a pattern documented in other lizard species. Mean T levels did not differ among morphs. However, yellow males maintained significantly higher T levels over time and displayed a stronger subsequent decline. Overall, in this species, seasonal T patterns differ among morphs, not mean values. Previous studies revealed that T suppresses the immune response; suggesting that a strong initial investment promoted by high T levels may trade-off against immunity (maintenance). Further experimental investigations are required to clarify the relationship between T and reproductive effort in polymorphic species that exhibit complex temporal pattern of T levels.

1. Introduction

Proximal mechanisms that underlie the maintenance of colour polymorphism (CP) within animal populations are involved in reproductive isolation and speciation (Huxley, 1955; West-Eberhard, 1989; Corl et al., 2010). The persistence of different colour morph subpopulations within a given population is generally associated with the coexistence of alternative reproductive strategies, each one involving specific trade-offs among behavioural, morphological, physiological, and other life history characteristics (Sinervo and Lively, 1996; Svensson et al., 2001; Sacchi et al., 2007a, 2009). Different morphs may represent alternative locally adapted optima within a single species (review in Sinervo and Svensson, 2002). Because the equilibrium between alternative strategies is not necessarily stable under changing conditions, some morphs can be locally favoured or may colonize novel environments whereas other may decline; thus, peculiar phenotypes can be fixed through allelic selection thereby promoting sympatric speciation ('morphic speciation'; see West-Eberhard, 1989; Corl et al., 2010).

Many reptile species contain populations with marked colour polymorphism and thus offer suitable models to investigate these issues. In lizards for example, distinct colour morphs coexist within populations (Thompson and Moore, 1991a,b, 1992; Carpenter, 1995; Sinervo and Lively, 1996; Sinervo and Zamudio, 2001; Olsson et al., 2007; Sacchi et al., 2007b), and may have a genetic basis (Thompson et al., 1993; Sinervo et al., 2001; Sinervo and Zamudio, 2001). In many cases, this colour polymorphism is associated with alternative reproductive strategies (Sinervo and Lively, 1996; Zamudio and Sinervo, 2000) and is stabilised by both natural selection (e.g. negative frequency-dependent selection,

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To bait or not to bait: it depends on the context

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Passive methods for sampling amphibians have been a well-established routine for long (Willson & Gibbons 2009). Bottle traps, funnel traps, and fish traps have proven to be effective, cheap and non-invasive methods for sampling amphibians under various environmental conditions (Griffiths 1985, Heyer et al. 1994, Dervo et al. 2014). Traps are usually not baited and rely on the spontaneity of newts to enter them. In one study, traps furnished with a light source yielded more captures than control traps without it (Grayson & Roe 2007), but another study found no difference between light-baited and unlit traps (Kröpfli et al. 2010). In a recent paper, Baker (2013) tested the hypothesis that traps baited with small cubes of stewing steak captured more individuals of *Lissotriton vulgaris* (Linnaeus, 1758) and *Triturus cristatus* (Laurenti, 1768) than bare traps. The author found clear support for this hypothesis, thus suggesting that baited traps are useful when sampling newts.

Even though the study by Baker (2013) tested 125 pairs of traps (baited and not baited) in seven ponds over seven years, not one single bycatch was reported. We define bycatch as all animals that could be casually trapped but are not the target species of a study (Hall 1996). It is reasonable to assume that syntopic small animals that rely upon chemical cues for foraging, may be lured to, and trapped in, baited traps. For example, the grass snake *Natrix natrix* (Linnaeus, 1758), a species that is widespread across Europe and predates upon amphibians and from which scavenging behaviour has been reported in the literature for this species (Ayres 2012), might be lured to traps containing possible prey items. Some invertebrates also predate upon amphibians, like water beetles (Lanza et al. 2007), and they rely on chemical and visual cues to find prey (Hodgson 1953, and references herein). If amphibian predators are present in the study area, as it is often the case (authors' unpubl. obs.), traps baited with small pieces of steak may attract scavengers, like some snakes and beetles (Ayres 2012). In such a case, the presence of bait could lead to unexpected results. We actually expect that the presence of predators inside traps may discourage amphibians from entering them. Moreover, if a predator enters the trap when some amphibians are already trapped, it could attempt to predate upon them. In such cases, the study results would likely be distorted, and therefore great caution should be exercised when planning a sampling protocol.

We tested the hypothesis that in a habitat with both amphibians, especially newts, and their predators, baited traps do not yield more captures because they will also attract predators like snakes and water beetles.

We selected a pond in the Groane Regional Park (Lombardy, northern Italy, 45°38' N, 9°6' E), in an area with partial anthropogenic disturbance. The pond has a surface area of approximately 400 m², but it is subject to seasonal fluctuations. The pond contains no fish and represents the most important site for amphibian reproduction in the area (Gatti & Sannolo 2014). Two newt species, *Lissotriton vulgaris* and *Triturus carnifex* and the anurans *Rana dalmatina* (Fitzinger in Bonaparte, 1839) *Rana latastei* Boulenger, 1879, *Pelophylax* kl. *esculentus* (Linnaeus, 1758), and *Hyla intermedia* Boulenger, 1882 reproduce in this pond.

We selected a period during which the two newts species are present in the pond, although *L. vulgaris* was already leaving it. By surveying the area in the same period the previous year, we verified that *N. natrix* and several species of Dytiscidae (Insecta: Coleoptera) were also present at the time of the study. On 10 May 2014, we set up six cylindrical funnel traps in the water along the shore of the pond. Traps were hand-made from PVC netting (50 × 21 cm; 3 mm mesh size; 175 g total weight), fitted with six funnels (10 cm external Ø, 3 cm internal Ø; Fig. 1). Three traps were baited with a small piece of ground beef (~5 g)

This is the end,
My only friend,

The end.

The Doors (1967)