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Developing, testing and deploying new tools and methods for ecophysiological and field research and conservation – an applied focus on squamates and crocodilians

Frederico Maria Chaves Fernandes Neto Barroso

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Doctoral Program in Biodiversity, Genetics and Evolution
Faculty of Sciences of the University of Porto
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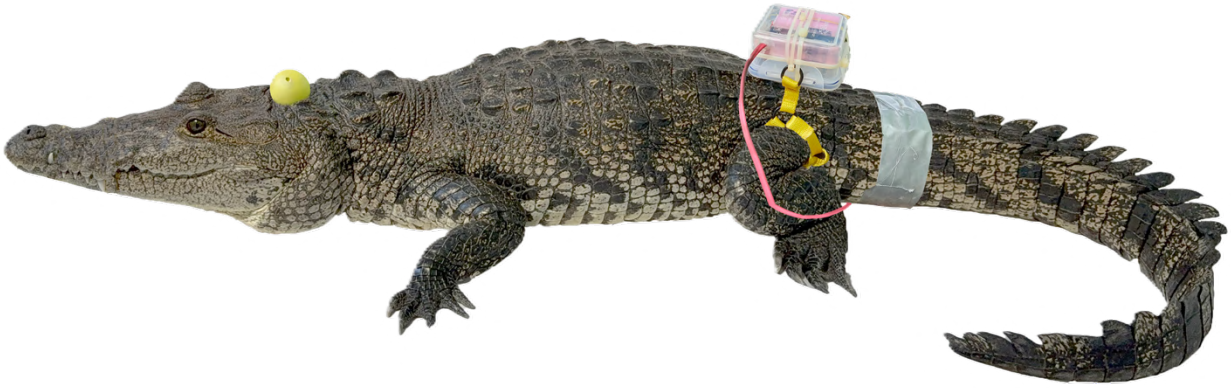

Developing, testing and deploying new tools and methods for ecophysiological and field research and conservation – an applied focus on squamates and crocodilians

Frederico Maria Chaves Fernandes Neto Barroso

Thesis carried out as part of the Doctoral Program in Biodiversity, Genetics and Evolution

Faculty of Sciences of the University of Porto

2026

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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 ou não 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.

A Faculdade de Ciências da Universidade do Porto foi a instituição de origem do candidato, tendo o trabalho apresentado nesta dissertação sido orientado pelo Doutor Miguel Ángel Carretero, Professor Auxiliar do *Departamento de Biologia da Faculdade de Ciências da Universidade do Porto* e Investigador do *Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO-InBIO)/BIOPOLIS, Program in Genomics, Biodiversity and Land Planning*, e co-orientado pelo Doutor Marco Sannolo, PhD, e pelo Doutor Mauricio González-Jáuregui, Professor e Investigador do *Centro de Desarrollo Sustentable Y Aprovechamiento de la Vida Silvestre* da *Universidade Autónoma de Campeche*, México. O trabalho foi realizado no CIBIO-InBIO/BIOPOLIS, como instituição de acolhimento, no âmbito do Programa Doutoral em Biodiversidade, Genética e Evolução (BIODIV).

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I dedicate this work to my great-grandparents, “Bivó” & “Bivô”,
as well as to all those other also loved and lost along the way.

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In October 2019, I entered the BIODIV programme with a definite and meticulously designed plan of action, a “bullet-proof” project idea (and title), and fully embracing the concept of a near-robotic and hyper-productive version of myself: “Fred 3.0”, built solely for productivity and the pursuit of perfect output, whatever the personal cost. Little did I know that my academic and personal path would take many unexpected turns. Along these detours, and through the people I met along the way, I came to rediscover something I now cherish even more: my own humanity and an honest acceptance of the imperfections it entails. It has been a journey longer than expected, but rarely lonely or dull. I am deeply grateful not only for reaching this important milestone, but for all the individuals and institutions that made it possible.

First and foremost, I thank Miguel Angel Carretero. More than a supervisor, Miguel has been what I can only describe as the best scientific and academic “grandfather” one could ask for. He has always been present whenever I needed support, and I will remain grateful for the immense intellectual freedom and trust he bestowed upon me to develop my own PhD project and the many parallel ideas that accompany it, who have shaped the scientist I am today. The ideas compiled in this thesis were built upon that trust. Beyond belief and encouragement, Miguel provided something even more essential: foundations. Through long conversations and brainstorming sessions, he helped mould the scientific grounding of this work, helping me solidify my place in the field of reptile thermal ecology. Equally important were the interpersonal foundations - his constant yet respectful encouragement to collaborate, explore, and engage beyond my immediate comfort zone was the catalyst behind the connections and many of the collaborations and colleagues - turn friends - featured in this thesis and these acknowledgements. For the trust, support and friendship that transcend formal supervision, I am profoundly grateful.

Yet, while Miguel provided the foundations, then Marco Sannolo taught me how to build upon them. I fondly remember when Marco himself was a PhD student under Miguel’s supervision - hence the “grandparent” reference - and I was a master’s student taking my first steps in reptile thermal ecology. Marco taught me how to walk in this field. The first time I held a FLIR T335 thermal camera - a tool central to this thesis - Marco showed me how to use it. The first lizard I noosed - a *Timon lepidus* living in the backroad wall between CIBIO and the greenhouses - Marco set up the noose and helped me free the animal. Many of the methods that inspired this methodological thesis trace back to those early lessons. I am grateful for Marco’s mentorship, continued supervisory support and

technical guidance, particularly in statistics, but even more for the strong friendship built over the years. The example he sets — professionally and personally — continues to inspire me to pursue ambitions beyond those of the original plan.

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The turbulence of doctoral life - amplified by a global pandemic, a period of loss, poorly-timed journey of self-improvement, and an engraved difficulty in finding work-life balance - were a stern test to my mental health. One to which I confess I was ill-prepared. I am therefore deeply grateful to Dra. Isabel Rasteiro for her support during the most difficult periods and for helping me find the tools to navigate toward brighter days. I also thank Dra. Luísa and Lola, the therapy dog, for their role in that process.

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Resumo

A medição de temperatura corporal é fulcral para o estudo da ecologia térmica em ectotérmicos. Desde o início da área de estudos da biologia térmica, no início/meados do século XX, as medições da temperatura corporal moldaram a nossa compreensão da termorregulação, da fisiologia, do desempenho fisiológico, do uso do habitat e da vulnerabilidade a alterações ambientais/climáticas. No entanto, muitos dos estudos fundadores que ainda hoje definem o campo basearam-se num conjunto metodológico relativamente limitado, assente maioritariamente em ferramentas de medição por contacto direto com o animal, como é o caso dos termopares/sondas cloacais e dataloggers simples, e volumosos. Ainda assim, estas abordagens estabeleceram conceitos fundamentais e têm permitido uma estandardização e manutenção de comparabilidade dos dados ao longo de décadas de investigação.

Nas últimas décadas, contudo, o rápido desenvolvimento tecnológico expandiu de forma significativa as ferramentas disponíveis para medir a temperatura e parâmetros fisiológicos relacionados. A termografia infravermelha, o vídeo radiométrico, os dataloggers miniaturizados e a telemetria permitem atualmente quantificar a temperatura com resoluções temporais e espaciais sem precedentes, tanto à escala do habitat como das diferentes regiões corporais do animal. Ainda assim, a investigação nesta área respondeu de forma bipolar a esta expansão tecnológica, oscilando entre a inércia metodológica e um entusiasmo tecnológico acrítico. Em alguns casos, os métodos tradicionais permanecem dominantes de forma pouco ponderada, apesar de limitações evidentes. Noutros, novas ferramentas são adotadas sem considerar a necessidade de calibrações que assegurem continuidade histórica e padronização de dados e metodologias.

Esta tese defende um meio-termo conservador: uma integração conscienciosa e calibrada das tecnologias emergentes nos quadros estabelecidos da ecologia térmica. Em vez de substituir medidas e metodologias tradicionais, as novas ferramentas devem ser validadas, contextualizadas e combinadas de modo a preservar a comparabilidade, ao mesmo tempo que reforçam a interpretação fisiológica e o realismo ecológico dos dados recolhidos.

O Capítulo 1 consiste numa introdução aprofundada ao campo da ecologia térmica dos ectotérmicos, contextualizando o estado da arte metodológico nesta área de investigação. Em seguida, ao longo dos quatro capítulos empíricos subsequentes,

desenvolvo, valido e aplico metodologias complementares com o objetivo de refinar a forma como a temperatura corporal é medida e interpretada em lagartos e crocodilianos.

O Capítulo 2 avalia a fiabilidade da termografia infravermelha como proxy não invasivo da temperatura corporal interna em lagartos. Ao quantificar a relação entre temperaturas superficiais e cloacais em condições laboratoriais padronizadas, este capítulo demonstra que a imagem termográfica pode fornecer estimativas robustas da temperatura interna e reforça a importância de considerar, através de calibração adequada, parâmetros como a emissividade e o contexto ambiental na inferência de temperatura interna. Assim, os resultados demonstram que a termografia pode constituir uma ferramenta não invasiva poderosa para inferir o estado interno de lagartos, quando ancorada em medições por contacto estabelecidas para efeitos de calibração.

O Capítulo 3 amplia a utilização da imagem térmica para além de medições pontuais simples, introduzindo o vídeo térmico radiométrico como ferramenta para quantificar, com elevada resolução espacial e temporal, taxas de fluxo térmico entre o animal e o seu ambiente. Apresenta todo um novo set-up experimental, modular e replicável, que permite dissociar as contribuições heliotérmicas e tigmotérmicas para o ganho e a perda de calor em pequenos répteis. Ao resolver simultaneamente as dinâmicas de temperatura em diferentes regiões corporais, esta abordagem evidencia a heterogeneidade inerente à “temperatura corporal” e demonstra que a troca térmica constitui um processo espacialmente estruturado e dependente do tempo, fortemente condicionado pela estratégia termorreguladora adotada.

Passando dos lagartos para os crocodilianos, o Capítulo 4 desenvolve e avalia protocolos para a monitorização a longo prazo da temperatura corporal interna utilizando dataloggers fixáveis e implantáveis, bem como telemetria. Aborda estratégias e locais de fixação dos loggers, considerações relativas à recuperação de dados e apresenta um quadro metodológico padronizado para a monitorização da temperatura interna em répteis de grande porte e semi-aquáticos.

Por fim, o Capítulo 5 aborda um aspecto frequentemente negligenciado nos estudos de ecologia térmica de répteis, mas metodologicamente crítico: a manutenção adequada e padronizada de répteis em cativeiro durante estados inativos (nomeadamente a brumação). Ao formalizar um protocolo de brumação replicável para duas espécies de lacertídeos em particular, este capítulo evidencia uma lacuna nas diretrizes de manutenção de ectotérmicos durante estados inativos, com implicações éticas e metodológicas subsequentes.

Por fim, o Capítulo 6 apresenta uma síntese final, extraindo os principais resultados e contributos de cada um dos quatro capítulos empíricos e discutindo a sua relevância para o avanço do campo.

No seu conjunto, o trabalho apresentado nesta tese contribui para: i) validar o uso da termografia infravermelha como extensão calibrada das medições tradicionais de temperatura por contacto em lagartos; ii) introduzir o vídeo radiométrico de alta resolução como ferramenta para quantificar dinâmicas espacialmente explícitas de troca de calor e dissociar experimentalmente as contribuições heliotérmicas e tigmotérmicas para a termorregulação em pequenos ectotérmicos; iii) estabelecer orientações anatomicamente informadas para a monitorização a longo prazo da temperatura interna em crocodilianos através de soluções de biologging; iv) formalizar um protocolo laboratorial replicável de manutenção de répteis durante estados inativos (especificamente a brumação em lagartos); e v) avançar um quadro metodológico integrador que reconcilia a comparabilidade histórica com a inovação tecnológica na ecologia térmica.

No contexto da aceleração da mudança ambiental, os modelos preditivos das respostas dos ectotérmicos dependem de parâmetros fisiológicos robustos e comparáveis. Uma integração cuidadosa de novas tecnologias - sem sucumbir à inércia conservadora nem ao progressismo acrítico - constitui o caminho mais fiável a seguir. Desta forma, refinar a forma como medimos a temperatura, bem como as variáveis a ela associadas, é, em última instância, refinar a forma como compreendemos e conservamos os organismos cuja biologia dela depende.

Palavras-chave: ecologia térmica, medição da temperatura corporal, desenvolvimento metodológico, termografia infravermelha, vídeo radiométrico, biologging, protocolos de brumação e de maneio animal, ectotérmicos.

Abstract

The measurement of body temperature lies at the core of ectotherm thermal ecology research. Since the emergence of modern thermal biology in the early-to-mid twentieth century, measurements of body temperature have shaped our understanding of thermoregulation, physiology, performance, habitat use and vulnerability to environmental change. Nevertheless, many of the early seminal studies that define the field to this day relied on a relatively limited methodological toolkit, largely based on contact measurement tools such as cloacal thermocouples and simple, yet bulky, dataloggers. These approaches established crucial foundational concepts and enabled comparability across decades of research.

Over the last few decades, however, rapid technological development has dramatically expanded the tools available to measure temperature and related physiological parameters. Infrared thermography, radiometric video, miniaturised dataloggers and remote telemetry now allow temperature to be quantified at unprecedented temporal and spatial (both at the scale of habitat and of the animal's body parts) resolutions. Yet the field has responded unevenly to this technological expansion, oscillating between methodological inertia and uncritical technological enthusiasm. In some cases, traditional methods remain uncritically dominant, despite clear limitations. In others, novel tools are adopted blindly, without sufficient calibration or consideration for historical continuity and standardisation.

This thesis argues for a conservative middle ground: a conscientious and calibrated integration of emerging technologies into established thermal ecology frameworks. Rather than replacing traditional measures, new tools should be validated, contextualised and combined in ways that preserve comparability while enhancing physiological interpretation and ecological realism.

Chapter 1, consists of an in-depth introduction to the field of ectotherm thermal ecology research, contextualising the methodological state of the art for the field. Then, across the four subsequent empirical chapters, I develop, validate and deploy complementary methodologies to refine how body temperature is measured and interpreted in squamates and crocodilians.

Chapter 2 evaluates the reliability of infrared thermography as a non-invasive proxy for internal body temperature in lizards. By quantifying the relationship between surface and cloacal temperatures under standardised laboratory settings, this chapter demonstrates

that infrared imaging can provide robust estimates of internal temperature and reiterates the importance of addressing emissivity and environmental context through appropriate calibration. Importantly, the results reinforce that thermography can be a powerful non-invasive tool to infer internal state when anchored to established contact measurements for calibration.

Chapter 3 advances the use of thermal imaging beyond simple point measurements by introducing radiometric thermal video to quantify rates of heat exchange at high spatial and temporal resolution. It introduces a novel, modular and replicable experimental setup which enables the decoupling of heliothermic and thigmothermic contributions to heat gain and loss in small squamates. By resolving temperature dynamics across different body regions simultaneously, this approach reveals the heterogeneity inherent in “body temperature” and demonstrates that thermal exchange is a spatially structured and time-dependent process that depends strongly on the thermal exchange strategy.

Shifting from squamates to crocodilians, Chapter 4 develops and evaluates protocols for long-term internal body temperature monitoring using attachable and implantable data loggers, as well as remote telemetry. It addresses attachment strategies and locations, considerations for data retrieval, and provides a standardised methodological framework for monitoring core temperature in large-bodied, semi-aquatic reptiles.

Chapter 5 addresses a frequently overlooked yet methodologically critical aspect of thermal research: the safe and standardised captive husbandry of lizards during natural inactive states (namely brumation). By formalising a replicable brumation protocol for two lacertid species, this chapter exposes a gap in husbandry guidelines for maintaining ectotherms during inactive states, with both ethical and downstream methodological implications.

Finally, Chapter 6 provides a closing synthesis extracting the main findings and outputs of each of the four empirical chapters and discussing their contribution to the field.

Collectively, the work presented in this thesis contributes towards: i) validating the use of infrared thermography as a calibrated extension of traditional contact-based temperature measurements in lizards; ii) introducing high-resolution radiometric video as a tool to quantify spatially explicit heat exchange dynamics and experimentally disentangle heliothermic and thigmothermic contributions to thermoregulation in small ectotherms; iii) establishing anatomically aware guidelines for long-term internal temperature monitoring in crocodilians through biologging solutions; iv) formalising a replicable laboratory husbandry protocol for maintaining reptiles during inactive states

(specifically brumation in lizards); and v) advancing an integrative methodological framework that reconciles historical comparability with technological innovation in thermal ecology.

In the context of accelerating environmental change, predictive models of ectotherm responses depend on robust and comparable physiological parameters. A careful integration of new technologies - neither conservative inertia nor uncritical progressiveness - offers the most reliable path forward. Refining how we measure temperature, and its associated variables, ultimately refines how we understand and conserve the organisms whose biology depends upon it.

Keywords: thermal ecology, body temperature measurement, methodological development, infrared thermography, radiometric video, biologging, brumation and husbandry protocols, ectotherms.

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$\partial T_{d\text{-}c}$	Temperature differential between dorsum and cloaca
$\partial T_{axilla\text{-}stomach}$	Temperature differential between axilla and stomach
$\%T_{br}$	Percent Thermal Performance Breadth
ANOVA	Analysis of Variance
BPC	*Biosistemas Productivos Cocodrilo*
CI	Confidence Interval
CCTV	Closed Circuit Television
CT_{max}	Critical Thermal Maximum
CT_{min}	Critical Thermal Minimum
CT_x	Critical thermal (maximum and/or minimum)
df *or* d.f. *or* deg. freed.	Degrees of freedom
d_b	Thermoregulatory Accuracy
EU	European Union
EWL	Evaporative Water Loss
E	Thermoregulatory Effectiveness
ε	Emissivity
FOL	Field of View Limiter
FPS	Frames Per Second
FOV	Field of View
GPS	Global Positioning System
h *or* hr	Hour
IR	Infrared
IRT	Infrared Thermography
I	Thermoregulatory Effectiveness
LRT	Likelihood Ratio Tests
LWIR	Long-Wave Infrared
LME	Linear Mixed-Effect [Model]
MDS	Multidimensional Scaling
NFC	Nearfield Communication Protocol
NTC	Negative Temperature Coefficient
OLS	Ordinary Least Squares

Pers. obs.	Personal Observation
Pers. comm.	Personal Communication
R^2	R-squared
RFID	Radio-Frequency Identification
RGB	Red Green and Blue [signal]
R&D	Research and Development
RMA	Reduced Major Axis
SVL	Snout-Vent Length
SD	Standard Deviation
SE	Standard Error
ST	Supplementary Table
Temp.	Temperature
T_b	Body temperature
TTL	Total Length
T_c	Cloacal Temperature
TPC	Thermal Performance Curves
T_{pref}	Preferred Body Temperature
T_{pant}	Panting Threshold
T_{gape}	Gaping Threshold
T_{opt}	Thermal Optimum
T_{set}	Thermal Set-Point Range
T_e	Operative Temperatures
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UVB	Ultraviolet B
VT_{max}	Voluntary Thermal Maximum
VT_{min}	Voluntary Thermal Minimum
VT_x	Voluntary Thermal (Maximum and/or Minimum)

1. Chapter 1 – General Introduction

1.1. Ecophysiological Principles of Thermoregulation: A Focus on [Non-Avian] Reptiles

1.1.1. Fundamentals of Thermal Biology: Ectothermy vs. Endothermy and the Role of Temperature in Life

Whether to regulate body parameters, such as temperature, or to adjust them to the environment is a fundamental question each organism must tackle at both an evolutionary and ecological scale (Bodensteiner et al, 2020). The chosen path (albeit often intertwined or interchangeable) will inevitably play a major role in shaping an organism's morphology, physiology and behaviour, ultimately contributing towards the shaping of its ecology and, ultimately, its evolutionary success (Angiletta, 2009; Angilletta, Niewiarowski, & Navas, 2002). Applied to temperature, this presents the alternative pathways of whether an organism's body temperature will simply track (i.e. conform) to environmental temperatures, or whether, through a combination of morphological, physiological and/or behavioural responses, the organism will attempt to regulate its body temperature, potentially maintaining it stable within a given range (i.e. thermal homeostasis) (Angilletta, 2009). Historically, this led to the emergence of two broad terms that define such seemingly bimodal strategies: thermoconformity vs thermoregulation (Huey and Slatkin, 1976). The reality, however, is inevitably more complex, as very rarely can an organism be qualified as either a perfect thermoconformer or a perfect thermoregulator. Instead, every living being will fall somewhere amidst a continuum of which these two strategies represent opposite extremes (Angilletta, 2009).

The realisation that such a continuum is a better description of what is observable in nature, led to the very human necessity to define a few more terms to label organisms into logical, yet mostly polyphyletic, functional groups. The concepts of poikilothermy vs homeothermy emerge as organisms whose internal body temperature varies extensively (due to an inexistent or weak attempt at thermoregulating), in contrast to those who maintain stable internal body temperatures (through thermoregulation), respectively (Crompton, Taylor, & Jagger, 1978; Seebacher, Grigg & Beard, 1999; Seebacher & Shine, 2004). Examples of poikilothermic species include many plants and amphibians, some reptiles, invertebrates and fish, and the naked mole-rats (Buffenstein & Yahav, 1991; Grigg, Beard, & Augee, 2004) and sloth (Britton & Atkinson, 1938). On the other hand, homeotherms are notoriously represented

by most mammals and birds (Grigg, Beard, & Augee, 2004) – although examples extend beyond these to include some teleost fish and some sharks (e.g. Goldman et al., 2004), dinosaurs (Gillooly, Allen, & Charnov, 2006) and even some extant reptiles (Paladino, O'Connor, Spotila, 1990). However, there are also many organisms that, either throughout their lives or for specific life-stages or periods (such as during torpor or in the reproductive season) may shift between one or the other strategy. These are referred to as heterotherms (Cooper & Geiser, 2007; Grigg, Beard, & Augee, 2004), and are represented in many taxonomic groups.

On the route to homeothermy, most organisms deviated from the path of thermoconformity, as environmental conditions often fall well outside conservative physiological optima (Angiletta, 2009; Crompton, Taylor, & Jagger, 1978; Grigg, Beard, & Augee, 2004). Nevertheless, it is important to point out that such route is no "one-way street", as the natural world is punctuated with multiple examples of thermoconforming organisms with thermoregulatory, or at least heterothermic, ancestors (e.g. the naked mole-rat - Daly et al., 1997). For most homeotherms, however, thermoregulatory strategies inevitably become a cornerstone of their physiology, influencing survival, behaviour, and overall ecological and evolutionary success (Angilletta, 2009). Within this strategy, two broad categories emerge again, depending on whether most of the heat required to thermoregulate is obtained from an external/environmental source (i.e. ectothermy), or from endogenous (e.g. metabolic) thermogenesis (i.e. endothermy) (Porter & Gates, 1969). These contrasting approaches reflect distinct evolutionary trade-offs, strongly shaping a species' physiological, behavioural, and ecological traits (Angilletta et al, 2009; Angilletta et al., 2010; Angilletta, Niewiarowski, & Navas, 2002). The dichotomy between these strategies underpins some of the diversity of life history traits and ecological niches observed across the tree of life (Angilletta et al., 2010; Angilletta, Niewiarowski, & Navas, 2002; Buckley, Hurlbert, & Jetz, 2012). Yet, as before, there are further layers of complexity within these categories, as some heterotherms are able to interchangeably navigate between these two strategies (e.g. Tattersall et al., 2016).

As mentioned above, ectothermy is defined by an organism's reliance on external (i.e. environmental) heat sources and sinks as means to regulate one's body temperature (Angilletta, 2009; Bogert, 1949) (Fig. 1.1A). For instance, most reptiles, as ectotherms, lack the ability to metabolically generate sufficient internal heat to maintain a constant body temperature. Instead, they engage behavioural and physiological mechanisms that modulate heat exchange with their environment (Huey, 1982). For example, a cold lizard may bask in the sun to absorb solar radiation (i.e. heliothermy) until it acquires a suitable (or preferred/optimal) body temperature. Alternatively, one that has surpassed such optimal

temperature, will instead retreat to a shaded area or colder microhabitat in order to cool down. In contrast, endotherms are able to maintain relatively stable internal body temperatures through the heat generated by metabolic processes (Grigg, Beard, & Augee, 2004; Legendre & Davesne, 2020) (Fig. 1.1B), yet this endogenous thermogenesis is still coupled with physiological mechanisms – many analogous to those observed in ectotherms (Grigg, Beard, & Augee, 2004) and controlled by the same brain regions (Boulant, 2000; Webb & Johnson, 1972; Webb, Johnson, & Firth) - that also modulate thermal exchanges within the body and its external environment. Nevertheless, these ultimately allow them to function somewhat independently of environmental conditions. Ultimately, this distinction has profound ecological and energetic implications that present cost and benefits to both strategies (Angilletta, 2009; Buckley, Hurlbert, & Jetz, 2012; Gillooly, Gomez, & Mavrodiev, 2017; Huey, 1982; Huey & Slatkin, 1976).

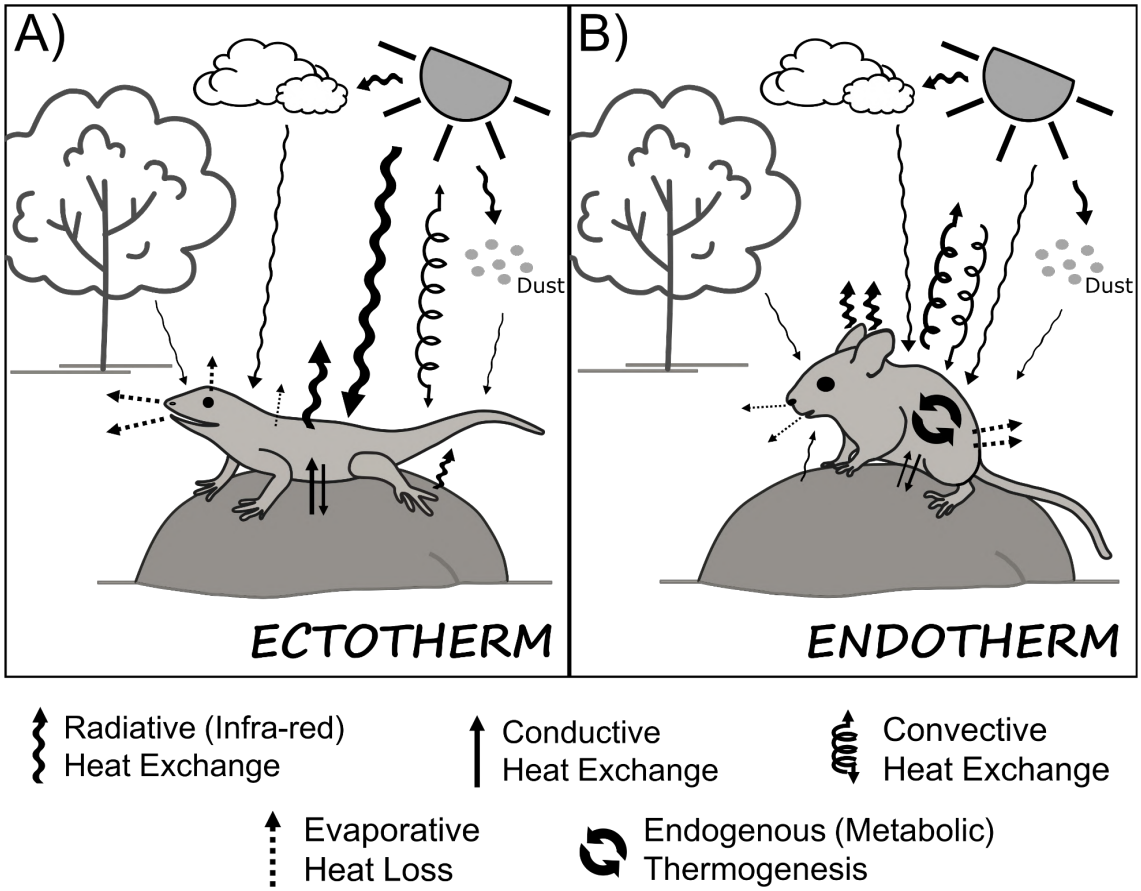

Fig. 1.1: Schematic representation of the main energy fluxes between organisms and their thermal environment. Conceptual diagram of the principal heat fluxes between an organism and its thermal environment. Arrows represent directionality (arrow direction) and relative proportion/importance (arrow thickness; symbolic) of the different thermal exchange routes at play: radiation, conduction, convection, as well as internally generated metabolic heat (in the case of endothermy; absent in ectotherm due to its [usually] negligible contribution towards T_b), and through heat loss to evaporation of water from cutaneous surfaces and mucosae. Solar radiation and longwave exchange with surrounding objects (e.g. substrate - represented by the ground, rock and tree; atmosphere - represented by the clouds and "dust" particles in suspension) all contribute to net radiative flux; contact with the substrate (represented by the rock) mediates conductive heat transfer; and wind/ air currents and thermal exchange with surrounding boundary layer mediates convective heat exchange. *Image credit: Cristiana I. Marques*

One of the most significant advantages of ectothermy is its energy efficiency (Huey, 1982; Huey & Slatkin, 1976). Ectotherms typically have metabolic rates that are an order of magnitude lower than those of endotherms of a similar size (Gillooly, Gomez, & Mavrodiev, 2017). This efficiency allows them to thrive in resource-poor environments where energy-intensive endothermy would be disadvantageous (Brown et al., 2004; Pough, 1980). The lower metabolic rates, and hence lower resource (i.e. food) requirements, also enable them to attain population densities and habitat carrying capacities that would be unsustainable for their endothermic counterparts (Brown et al., 2004). For instance, ectotherms can survive

prolonged periods of food scarcity by reducing even further their metabolic activity; an adaptation that has allowed reptiles to colonize arid deserts, nutrient-poor forests, even minute islands/islets and other challenging habitats (De Souza et al., 2003; Gavira et al., 2018; McCue, 2007). Additionally, the ability to match activity levels to favourable environmental conditions enables ectotherms to optimize energy expenditure, even at short temporal scales (Sears & Angilletta, 2015).

However, an ectotherm's reliance on environmental heat sources also imposes significant costs and constraints. Ectotherms are restricted in their activity patterns, often being limited to specific times of day or seasons when temperatures are suitable for activity or, at least, conducive to thermoregulation (Andersson, 2003; Angilletta, 2009). This dependency makes them highly vulnerable to fluctuations in environmental conditions, such as sudden cold spells or heat waves, which can significantly increase the cost of thermoregulation (e.g. Withers & Campbell, 1985). For example, cold temperatures can immobilize reptiles, leaving them susceptible to predation or reducing their ability to forage and reproduce effectively (Downes, 2001; Herczeg et al., 2007; Huey & Slatkin, 1976). Furthermore, another inherent cost of thermoregulation lies in the significant trade-off between time spent thermoregulating (to achieve optimal temperatures) and time spent performing activities that will maximise an individual's fitness (e.g. foraging, reproducing, guarding territory, etc.) (Angilletta, 2009; Huey, 1982; Huey & Slatkin, 1976). Inevitably, the latter relies on the former, as an animal's performance in such fitness-increasing endeavours will be maximised by performing these at an optimum body temperature. Still, an animal will often only be able to do one or the other (thermoregulate vs feed/reproduce/fight/etc.) at any one point in time. Similarly, seasonal changes in the environment also impose strict limits on the distribution of ectothermic species, theoretically confining them to regions where temperatures remain within their tolerance ranges for sufficient durations (Grant & Porter, 1992). Furthermore ectotherm activity patterns are also spatially constrained as thermoregulatory efforts are heavily dependent on the extent of habitat thermal heterogeneity and on whether the scale of such heterogeneity still allows the animal to shuttle between thermally optimal and sub-optimal microhabitats without having to pay too heavy a cost in terms of time, energy and exposure to predation (Sears & Angilletta, 2015; Sears et al, 2016). Despite all this, ectotherms (reptiles in particular) still overfill the geographic distribution that would be theorised from purely considering the overlap of environmental conditions and their tolerance limits (Andersson, 2003; Camacho et al., 2024). This is largely due to their ability to behaviourally thermoregulate, but also through the very low metabolic cost of maintaining prolonged inactive states (e.g. brumation, aestivation) during thermally sub-optimal periods (Buckley, Rodda, & Jetz, 2008; Patterson & Davies, 1978).

In contrast, endothermy offers significant benefits in terms of ecological flexibility and independence from environmental temperatures (Clarke & Pörtner, 2010). Endotherms can remain active across a wide range of thermal conditions, and exploit diverse habitats, from the polar regions to hot deserts (Clarke & Pörtner, 2010). Their ability to maintain constant (within a narrow range) optimal body temperatures, resulting in high and stable metabolic rates, maximises and sustains high-levels of performance, across a range of environmental conditions (Clarke & Pörtner, 2010), thus supporting sustained activities such as long-distance migration, extended parental care, and high-intensity physical activity (e.g. during hunting or escaping predators), even in thermally adverse conditions (Bennet & Ruben, 1979). This higher metabolic independence allows endotherms to occupy ecological niches less accessible to ectotherms (Bennet & Ruben, 1979). However, these advantages come at a very high energetic cost (Bennet & Ruben, 1979). The constant need to generate heat requires endotherms to consume large amounts of food, which can be a significant constraint in resource-limited environments (Shankar et al., 2019). Moreover, the high metabolic demands of endothermy increase vulnerability to starvation (McNab, 2019) and impose substantial reproductive costs, as raising offspring requires significant energy investment (Koteja, 2000). The higher metabolism and energy expenditure observed in endotherms inevitably often culminates in increased rates of oxidative stress (Jastroch & Keuper, 2025), often resulting in faster senescence and comparably shorter life-spans (Reinke et al., 2022) (Fig. 1.2). Thus, while endothermy expands ecological possibilities, it also implies a series of trade-offs that limit its efficiency in certain environments and ecological contexts.

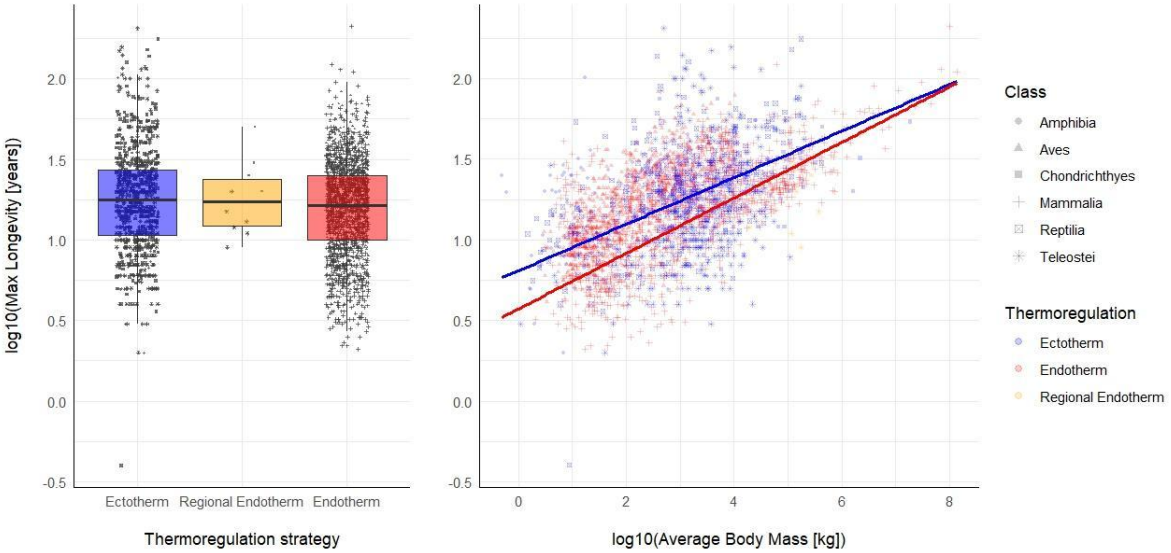

Fig. 1.2: Plots comparing the maximum longevity of 2934 vertebrate species. Maximum longevity was corrected for body mass and fitted linear mixed models considers Class as a random variable. Data on maximum longevity and body size obtained from Kuparinen, Yeung, and Hutchings (2023a, 2023b). Each vertebrate was subsequently individually characterized based on the main thermoregulatory strategy and disregarding hibernation/estivation/torpor states. The strategy of "regional endotherm" relates to species, such as some sharks, who are able to maintain some level of homeothermy in specific parts of their body, often through a combination of specific dynamic action and/or counter-current heat exchange systems (Bernal et al., 2003; Dolton et al., 2023). However, such are not cases of generalised, metabolic homeothermy.

Hence, considering the aforementioned costs and benefits of both strategies, it begs the question as to whether intermediate strategies (e.g. a facultative endotherm/ectotherm - a "homeothermic ectotherm" or a "heterothermic endotherm") should exist, and be ecologically successful. In short, the answer to this question is that they did (and still do) exist (e.g. echidnas - Grigg, Beard, & Augee, 2004), and have been/are successful! Yet, this is no evolutionary "silver bullet" either as each strategy is still bound to its particular trade-offs that bring inherent costs. For example, many mammals (endothermic) that inhabit seasonally cold environments will resort to heterothermic hibernation during winter (Ruf & Geiser, 2014). At this stage, animals will enter an inactive state that is characterised by rest phases (torpor bouts) interspaced with shorter periods of arousal (Horii, Shiina, & Shimizu, 2018; Ruf & Geiser, 2014). During such torpor bouts, the animal's metabolism drops drastically, along with its body temperature, to levels compared to those of ectothermic animals (Horii, Shiina, & Shimizu, 2018; Ruf & Geiser, 2014). However, during the arousal phases, body temperature, and hence metabolic rates, return to endothermic levels, yet the animal is still inactive and not feeding (Horii, Shiina, & Shimizu, 2018; Ruf & Geiser, 2014). Additionally, throughout both

these phases, as the animals remain largely inactive, they will not be performing fitness-increasing activities. They will also be reliant on accumulated energy reserves, which will be slowly but continuously drained (mostly by the arousal phases) until emergence from hibernation (Horii, Shiina, & Shimizu, 2018; Ruf & Geiser, 2014).

The above examples demonstrate that temperature is frequently a key factor in shaping the physiology, behaviour, and distribution of ectotherms. Virtually all physiological processes are temperature-dependent (Angilletta, Niewiarowski, & Navas, 2002; Huey, 1982), as illustrated by the thermal performance curves (TPCs) of most metabolic and physiological processes. These curves describe the relationship between body temperature and physiological performance, showing a distinct range where enzymatic activity, protein and cell-membrane function/stability, muscle contraction, and metabolic efficiency are optimized (Angilletta, 2009). Outside this optimal range, performance declines rapidly (Angilletta, 2006; Arnoldi et al, 2025), underscoring the critical role of thermoregulation in maintaining general homeostasis.

For many reptiles, the ability to regulate body temperature through behavioural strategies is essential for survival. By seeking out specific microhabitats or adjusting their body posture, shape or colour, reptiles can exploit environmental heterogeneity to achieve their preferred body temperatures (T_{pref}) (Bauwens, Hertz, & Castilla, 1996). For instance, heliothermic lizards bask in sunlight to rapidly increase their body temperature, while thigmothermic species rely on conductive heat from warm substrates to do so, with many species combining or interchanging between both strategies depending on time (available to bask, or time of day), microhabitat or opportunities available (Belliure & Carrascal, 2002; Sears et al., 2016). An array of behaviours allow reptiles to maximize physiological efficiency while minimizing energy expenditure (Angilletta, 2009). However, the effectiveness of these strategies is contingent on the availability of suitable heterogeneous thermal environments, making habitat selection a critical aspect of reptile ecology (Sears et al., 2016).

The interplay between thermal biology and a habitat's thermal landscape, at several scales, is particularly evident in the geographical ranges of ectotherms (Pincebourde et al., 2016). Species in more thermally stable environments, such as tropical rainforests, often exhibit narrow thermal tolerance ranges and either very specialized thermoregulatory behaviours or, alternatively, thermoconformity to the stable environmental conditions (Hertz, 1974; Huey, 1982; Huey et al, 2009; Morris & Rollinson, 2025). In contrast, those in variable climates, such as deserts or temperate zones, have broader tolerances and exhibit greater behavioural plasticity or seasonal acclimation to be able to cope with fluctuating or extreme conditions

(Angilletta, 2009). This variation highlights the adaptive significance of thermal biology in determining the abundance, diversity, and evolutionary success of ectotherms.

As global temperatures rise, understanding the distinctions between ectothermy and endothermy, and their associated costs, becomes increasingly important for predicting species responses to climate change. Ectotherms are particularly vulnerable to shifts in thermal regimes (not only average trends but also its variation and extremes - Morris & Rollinson, 2025; Schwanz, Hodgson, & May, 2018), as their physiological and behavioural adaptations are tightly linked to environmental conditions. Reduced thermal safety margins and loss of [thermally suitable] habitat are likely to exacerbate the challenges faced by many ectothermic species (Huey et al., 2009), emphasizing the need for conservation strategies informed by thermal ecology.

1.1.2. Thermoregulatory Strategies in Reptiles: Behaviour, Physiology, and Anatomy

Reptiles exhibit a suite of interconnected thermoregulatory strategies that are critical to their survival and ecological success. These strategies span behavioural adjustments, physiological mechanisms, and anatomical adaptations (Angilletta, 2009; Huey, 1982; Seebacher & Franklin, 2005), each contributing to their ability to maintain optimal body temperatures in the face of environmental variability. Unlike endotherms (which rely extensively on internal metabolic heat), reptiles employ a finely balanced interplay of external and internal strategies to navigate their thermal environments. This integration allows them to thrive in environments ranging from the arid deserts to the wet tropical rainforests and even the open ocean, demonstrating their evolutionary adaptability and resilience.

Behavioural thermoregulation is the first and most visible strategy to manage thermal extremes in reptiles allowing them to control their exposure to heat and cold with remarkable precision**.** Basking is perhaps the most obvious behaviour, enabling reptiles to efficiently absorb solar radiation. Heliothermic species, such as many diurnal lizards, actively seek sunlit surfaces in the morning to raise their body temperatures quickly after a cool night (Bawens, Hertz, & Castilla, 1996). This rapid warming allows them to activate critical physiological processes, including digestion, immune function, gestation and locomotion (Seebacher & Franklin, 2005). Without this behaviour, they would be restricted to periods of suboptimal performance, increasing their vulnerability to predators and reducing foraging efficiency and overall fitness (Carrascal et al., 1992).

In cold environments, basking is not merely beneficial, but a necessity. Studies on *Sceloporus spp.* lizard populations across elevational clines reveal that prolonged basking sessions are essential for lizards from high-elevation habitats to offset low overnight temperatures, ensuring that reproductive and metabolic processes remain functional (Adolph, 1990). The relevance of this strategy is further demonstrated by many strictly montane lizard species, such as *Iberolacerta monticola* and *Iberolacerta cyreni,* who are notorious for their ability to thermoregulate very efficiently through basking (Aguado & Braña, 2014; Ortega, Mencía, & Pérez-Mellado, 2017). This ability is key in enabling them to optimise their performance during the relatively short periods (daily and seasonal) when conditions are suitable for their activity. Similarly, desert-dwelling lizards, such as the bearded dragon (*Pogona vitticeps*), use basking to optimise thermoregulation during early morning hours before retreating to shaded areas as daytime temperatures peak above their tolerance thresholds (Bradshaw & Main, 1968). These behaviours illustrate how basking is carefully calibrated to environmental conditions, maximizing heat gain while minimizing energy expenditure, exposure to predators, heat stress and hydric stress.

Postural adjustments further enhance the effectiveness of basking and heat management (Rangel-Patiño et al., 2020; Stanton-Jones, Parusnath, & Alexander, 2018). By flattening their bodies, reptiles maximize the surface area exposed to sunlight, optimizing heat absorption. Conversely, coiling (e.g. in snakes) or compacting the body reduces surface exposure and surface-area-to-volume ratio, thus aiding to conserve heat during cooler periods (Ayers & Shine, 1997; Lillywhite, 1980). Similarly, some authors have suggested that, analogously to what is observed in several endotherms (e.g. penguins - Gilbert et al., 2006), some reptiles aggregate in order to decrease the rate of heat loss (Iryshkov et al., 2024; Reiserer, Schuett, & Earley, 2007; Shah et al., 2003), although alternative explanations are also hypothesised for such gregarious behaviour (Graves & Duvall, 1995; Khan, Richardson, & Tattersall, 2010). Furthermore, many chelonians are known to position their shell strategically while basking, angling it to maximize heat absorption or limit heat loss as required (Boyer, 1965). This strategy is also widely observed in many heliothermic lizards and crocodilians who often also orient their dorsum towards the sun to maximise their heating rates (Stanton-Jones, Parusnath, & Alexander, 2018). Crocodilians, in particular, are even able to do this while still partially submerged in water, by inflating their chest and adopting a “high-float” posture that exposes a large portion of the highly vascularized dorsum out of the water (Seebacher, 1999; Smith, 1975, 1979).

Desert-dwelling species, such as *Phrynosoma* spp. and *Sceloporus spp.*, must respond to dramatic daily temperature fluctuations and hence exhibit a high degree of postural plasticity

(Rangel-Patiño et al., 2020). By altering their body orientation relative to the sun (parallel vs perpendicular) and substrate (flat vs lifted), these lizards can modulate heat exchange efficiently (Rangel-Patiño et al., 2020). During midday, when temperatures soar, they often adopt a standing posture to reduce contact with the hot substrate, minimizing conductive heat gain (Rangel-Patiño et al., 2020). Contrastingly, many nocturnal and crepuscular geckos adopt the opposite strategy and will flatten their bodies against the underside of a warm rock – their shelter during the day - in order to raise their body temperature by conduction (i.e. thigmothermy) without the need to expose themselves to the sun (and predators) directly (Kearney & Predavec, 2000; Pereira et al., 2019; Vasconcelos, Santos & Carretero, 2012). This dynamic interplay of posture, incident solar radiation and substrate interaction exemplifies the fine-scale control reptiles exert over their thermal exchanges with the immediate environment.

Beyond basking and postural adjustments (which modulate mostly whole-body thermal exchange rates), gaping and panting are able to provide further fine-tuned control of [localised] heat loss and thus help prevent heat stress (Spotila Terpin, & Dodson, 1977). Upon reaching certain upper thermal thresholds (i.e. T_{pant} – panting threshold; T_{gape} – gaping threshold,), sometimes even while continuing to bask, many lizards and crocodilians, will also employ gaping (open mouths) or panting (open mouth with deep exhaling), which potentiate evaporative cooling mechanisms from the wet surfaces of the mouth (gaping and panting) and lungs (panting) (Tattersall, Cadena, & Skinner, 2006). Through these mechanisms the animal is able to protect sensitive organs, such as the brain (Webb & Johnson, & Firth 1972), from heat stress while other, less sensitive, body parts (e.g. the dorsum) continue to acquire heat during favourable basking opportunities (Tattersall, Cadena, & Skinner, 2006). This inevitably results in different body parts concurrently exhibiting different temperatures; a phenomenon known as regional heterothermy (Crawford. 1972; Heath, 1964), which is now known to occur in reptiles of all shapes and sizes (Barroso et al., 2016; Cox et al., 2023, 2024; Sannolo et al., 2014).

Additionally, reptiles also rely on microhabitat selection to manage their thermal environment (Bauwens, Hertz, & Castilla, 1996). This behaviour allows them to exploit the spatial and temporal variability of thermal landscapes. For instance, small lizards are known to shuttle between open, sunny areas and shaded refuges throughout the day to precisely maintain optimal body temperatures, a behaviour typical of many heliothermic species (Díaz & Cabezas-Díaz, 2004). In tropical environments, anoles selectively modify their perch height and microhabitat (perching on tree trunks vs branches) depending on light availability, to carefully balance thermal requirements with predator avoidance (Díaz-Marín et al., 2021;

Hertz, Fleishman, & Armsby, 1994). Meanwhile, some xeric reptiles, such as fringe-toed lizards (*Uma spp.*), burrow beneath sand during the hottest parts of the day, using the insulating properties of the substrate to avoid lethal temperatures (Pough, 1970). Others, such as the Mediterranean spiny-footed lizard (*Acanthodactylus erythrurus*) will carefully orientate their shallow underground burrows towards South, maximising early-morning warm-up while minimising the effect of mid-day heat (Seva, 1982) and eliminating the reliance on basking (Carretero & Llorente, 1995), as it can be [thermally/ physiologically] dangerous and [hydrically] costly basking in mesic and xeric habitats. Some geckos, on the other hand, such as several species of the genus *Tarentola* inhabiting the Sahara, Canary Islands and Cape Verde (Pereira et al., 2019; Vasconcelos et al., 2012), will carefully select a suitable rock to lay under during the day. Size in this rock selection process is key; too small of a rock and it may heat up beyond the gecko's thermal tolerance, forcing it to venture out in daylight to look for a new refugium – risking predation, overheating and dehydration. Too large, and the rock may never reach the gecko's optimal temperature range, compromising its performance when the gecko's active period starts, in the evening. Similar retreat site selection consequences have also been observed in snakes (Huey et al., 1989).

The thermal heterogeneity of habitats plays a crucial role in facilitating these behaviours, providing opportunities to mitigate the risks associated with extreme environmental conditions (Basson et al., 2016; Rangel-Patiño et al., 2020). Furthermore, familiarity with the [thermal] environment also plays an important role in mitigating the costs and risks of thermoregulatory endeavours, with studies showing that reptiles take advantage of the previous knowledge of the thermal heterogeneity of a given habitat to improve their thermoregulatory precision (Chelazzi & Calzolai, 1986). For crocodilians, turtles and even some squamates, microhabitat use extends to aquatic environments. By submerging in water, they exploit the thermal inertia of aquatic systems to buffer against rapid temperature fluctuations (Seymor, 1982), or resort to the water's higher thermal conductivity to cool-off when reaching close to the upper limits of their thermal preference during peak heat hours (Seebacher & Grigg, 1997; Smith, 1979). Many crocodilians have been shown to move between land and water to regulate body temperature, or even a combination of the two - the "high-float" posture (Seebacher, 1999; Smith, 1975) - with fine-scale behavioural and physiological adjustments ensuring optimal internal thermal conditions for activity (Johnson, Webb, & Tanner, 1976; Seebacher, 1999; Smith, 1979).

While behaviour provides immediate and flexible thermoregulatory control, physiological mechanisms enhance these actions (Angilletta, 2009; Huey, 1982) by directly influencing heat exchange with the environment and its subsequent redistribution across the body, thus

modulating internal thermal balance (Seebacher & Franklin, 2005). Circulatory adjustments, through vasomotor control for example, are central to this process (Bartholomew & Tucker, 1963; Grigg & Alchin, 1976; Seebacher & Franklin, 2004). Vasodilation of peripheral vessels increases blood flow to the skin, enhancing heat absorption or dissipation depending on environmental conditions (Baker, Weathers, & White, 1972; Clarac et al., 2017; Clarac & Quilhac, 2018). Conversely, vasoconstriction limits blood flow to peripheral tissues, conserving core body heat during cooler periods (Baker, Weathers, & White, 1972; Dzialowski & O'Connor, 1999, 2004). Hence, peripheral tissues often exhibit broader thermal ranges than the animal's core, resulting in frequent surface-to-core temperature gradients (Porter & Gates, 1969).

Crocodilians, for instance, display particularly advanced circulatory shunting mechanisms which may have important repercussions on the distribution and exchange of heat across the animal's body (Seebacher & Franklin, 2007). For instance, they can redirect blood flow between systemic and pulmonary circuits (via the foramen of Panizza) which, in addition to optimizing oxygen distribution during diving, may also have thermal distribution consequences (Grigg & Kirshner, 2015). Furthermore, during basking, increased blood flow to the skin, and particularly to the highly vascularised osteoderms (Clarac et al., 2017; Clarac & Quilhac, 2018), allows crocodiles to absorb solar radiation very efficiently. At night, on the other hand, reduced peripheral circulation conserves heat in their core. This dual function of the circulatory system highlights the evolutionary sophistication of physiological thermoregulation in reptiles.

Additional cardiovascular mechanisms further enhance the thermoregulatory benefits of such vasomotor control of blood flow. Many reptiles exhibit heart rate hysteresis whereby the heart rate of the animal will be higher during heating than during cooling (Zaar, Larsen, & Wang, 2004). This ensures higher cardiac output during heating which, combined with the aforementioned control of cutaneous perfusion, maximises heating rates of a warming reptile, with the reverse pattern observed during cooling (Bartholomew & Lasiewski, 1965; Franklin & Seebacher, 2003; Zaar, Larsen, & Wang, 2004). Similarly, semi-aquatic reptiles such as the marine iguana (*Amblyrhynchus cristatus*) will show an analogous response when diving into [cold] water. While submerged, they enter a state of bradycardia, drastically reducing their heart rate to around a third of that when resting on land (at optimal temperature) (Bartholomew & Lasiewski, 1965). Combined with cutaneous and peripheral vasoconstriction, this allows these medium-sized lizards to minimise their heat loss during their subaquatic foraging endeavours.

Hormonal regulation also plays a significant role in thermoregulation (Seebacher & Franklin, 2005). Stress-related hormones such as adrenaline can increase metabolic rates, temporarily elevating body temperature (Gupta & Thapliyal, 1983), as well as change heat distribution across the body by modulating heart rate (Seebacher & Franklin, 2001). Similarly, melatonin and thyroid hormones modulate long-term metabolic adjustments and affect the daily rhythm of body temperature, supporting thermoregulation under varying environmental conditions (Seebacher & Franklin, 2005; Tosini & Menaker, 1996). In gravid female reptiles, for example, hormonal changes facilitate the regulation of embryonic development, optimizing thermal conditions for successful reproduction (Đuričić et al., 2024). For instance, some species increase their basking behaviour to maintain elevated body temperatures that benefit embryonic development (Shine, 1980). However, these elevated body temperatures will incur a higher energetic and oxygen demand on the mother (Shine, 1980). Hence, others, such as *Zootoca vivipara,* will adopt the opposing strategy and select lower body temperatures when gravid (Carretero et al., 2005). Thus, the former strategy will provide optimal conditions for development of the progeny (increasing their rate of development and chances of survival), at the expense of the mother performing sub-optimally while gravid; while the latter strategy will optimise the mother's performance (increasing her chance of survival and future reproductive events) at the cost of slower or sub-optimal development of the progeny (Shine, 1980). Both strategies incur and tradeoff, and hence have associated costs and benefits, but ultimately both routes may effectively increase the mother's total reproductive output (and hence, her fitness) (Shine, 1980).

Furthermore, although [most] extant reptiles lack the sustained endogenous heat production seen in endotherms (but see, for example: Harlow & Grigg, 1984; Tattersall et al., 2016), metabolic processes, such as prolonged muscle activity (Harlow & Grigg, 1984) and digestion (Tattersall et al., 2004; Wang, Busk, & Overgaard, 2001), generate heat as a by-product. This "collateral thermogenesis" - known as "specific dynamic action" (Secor, 2008; Wang, Busk, & Overgaard, 2001) — while usually small in magnitude, may nonetheless still provide some transient thermal benefits to the otherwise ectothermic animal. Nevertheless these "small" thermal contributions scale up considerably with mass, with different authors estimating different [mass] thresholds (ranging from 5kg to ~10kg to 100kg - Smith, 1979; McNab, 2002; Bartholomew & Tucker, 1964; respectively) above which these could incur significant thermal gains to the ectotherm. Indeed, these may even be a relevant contributor to the level of homeothermy observed in large extant lizards (e.g. some varanids), chelonians, crocodilians (Klein et al., 2003; Turner & Tracy, 1985; Sato et al., 1994) and has even been hypothesized as a possible explanation for dinosaur homeothermy (Seebacher, Grigg & Beard, 1999).

Hence body size is an undeniably critical factor affecting thermal exchanges, as larger-bodied reptiles inherently exhibit greater *thermal inertia* (Bell, 1980). This term describes a body's resistance to change its temperature. Simply put, the larger the mass, the more energy is needed to cause the same thermal change. Thus, having a larger mass will effectively slow the rates of both heat gain and loss, providing a buffer against rapid environmental temperature fluctuations (Bell, 1980; Colbert et al., 1946; Seebacher, Grigg, & Beard, 1999). Taken to the extreme (e.g. large crocodiles and dinosaurs - Grigg & Kirshner, 2015; Seebacher, Grigg, & Beard, 1999; Spotila et al., 1973), very large ectothermic animals can rely on their large thermal inertia to maintain a constant body temperature. This form of [ectothermic] inertial homeothermy (McNab, 1978) is often referred to as gigantothermy (Paladino, O'Connor, & Spotila, 1990) and can still be seen in a few extant reptiles such as large chelonians (Paladino, O'Connor, & Spotila, 1990) and crocodiles (Seebacher, Grigg, & Beard, 1999; Spotila, Soule, & Gates, 1972). Furthermore, the thermal buffering advantage of increased body size is also implicit in the well-known Bergmann's rule (Bergmann, 1847). This ecogeographical relationship between body size and ambient temperature hypothesizes that animals living in colder environments (often associated with higher latitudes and/or altitudes) will attain larger body sizes than their warmer environment counterparts (even of the same or closely related species) (Meiri, 2010). Yet, while there is plentiful support for this rule in endotherms, the case for ectotherms is less well-founded, or at least divisive (Adams & Church, 2009; Ashton & Feldman, 2003; Meiri, 2010; Pincheira-Donoso, Hodgson, & Tregenza, 2008). Still some reptile species – namely the tortoise *Testudo graeca* (Werner et al., 2015) as well as several species of lacertid (Volynchik, 2014; Zamora-Camacho, Reguera, & Moreno-Rueda, 2014) and sceloporine (Angilletta et al., 2004) lizards as well as some geckos (Penniket & Cree, 2015) - have been tentatively shown to follow this rule.

However, the large inertia associated with gigantotherms may also result in an increased risk of overheating in hot climates (Seebacher, Grigg, & Beard, 1999), especially as larger shelters are harder to find and moving between thermal patches becomes increasingly difficult or costly for habitat thermal heterogeneity at such a large scale (Cloudsley-Thompson, 1991). Thus they require additional strategies such as shade-seeking behaviour, aquatic or amphibious lifestyles, or utilizing evaporative cooling mechanisms or vascular heat exchangers, in order to maintain their body temperatures within an optimal range (Seebacher, Grigg, & Beard, 1999). Smaller reptiles, by contrast to their giant counterparts, experience rapid thermal changes, can easily move between thermal patches, have multiple shelters available and must rely more heavily on behavioural strategies, usually focusing more on maximising heat gain rather than heat loss (Bartholomew & Lasiewski, 1965).

An additional wide array of anatomical adaptations further enhance a reptile's ability to thermoregulate. These may modulate heat exchange rates with the environment, heat distribution across the body, or both (Dzialowski & O'Connor, 1999 & 2001; Porter & Gates, 1969). For instance, the presence of large venous sinuses (e.g. sinus orbitalis) immediately adjacent to prime sites of evaporative cooling - such as the eye, mouth and snout - in the head of many lizards (e.g. lacertids - Bruner, 1907; and iguanids - Porter & Witmer, 2016), likely provides an efficient mechanism of cooling local blood flow, thus preventing overheating of the brain (Porter & Witmer, 2016). Another example includes the extensively ornamented micro-structure of crocodilian osteoderms (Clarac et al., 2017; Clarac & Quilhac, 2018). Their external structure is riddled with pits and grooves which are highly vascularised, as is their inner layer (i.e. the spongiosa) (Clarac et al., 2017). Combined, the convoluted micro-structure and its extensive blood perfusion make these bony-structures excellent sites of thermal exchange between the animal and its environment (Clarac et al., 2017). Finally, it is important to note the possible presence of counter-current heat exchange systems that may aid in preventing heat loss (or gain, in a thigmothermic context) from peripheral structures (e.g. limbs, tail) while maintaining optimal blood supply (i.e. no vasoconstriction needed) to said parts (Davenport et al., 2015; Tattersall, Cadena, & Skinner, 2006). This may be particularly important in animals that have specialized appendages, such as the adhesive lamellae of geckos, whose function must not be disregarded at the expense of thermoregulatory requirements. Ultimately, these features may often result in thermal windows, which not only present important sites of thermal exchange, but have the potential to be easily accessible surface proxies of core body temperature (Barroso et al., 2016; Porter & Witmer, 2016).

Additionally, skin properties, including texture and pigmentation (and hence, reflectance), can also significantly influence rates of heat exchange (Bogert, 1949). Lighter skin tones reflect solar radiation, reducing heat gain in high-temperature environments. Conversely, darker (e.g. more melanised) pigmentation enhances heat absorption, which is advantageous for basking species in cooler climates, such as those at altitude (Clusella-Trullas, Van Wyk, & Spotila, 2007). Indeed, many species of reptiles inhabiting higher altitudes and latitudes (or populations of species that range over latitudinal or altitudinal clines) tend to be darker or even fully melanic (Clusella-Trullas van Wyk, & Spotila, 2007 & 2009; González-Morales et al., 2024). One long-standing theory for this, known as the thermal melanism hypothesis (Porter & Norris, 1969), suggests that such darker forms are positively selected for in such environments as they enable higher rates of radiant heat gain in these otherwise thermally restrictive habitats. Nevertheless, other non-thermoregulatory (Hastings et al., 2023) explanations can also account for darker skin coloration, such as increased UV protection

(Goldenberg et al., 2024), given the higher UV intensity also associated with some of these habitats (e.g. at high altitude).

Furthermore, many species are capable of physiological colour change. This change in colour may happen as fast as in a few seconds or minutes (Hu et al., 2026) – such as in some geckos (e.g. *Tarentola mauritanica* - Vroonen et al, 2012; and chameleons - Ligon & McGraw, 2013) – or slow, taking place over seasons (e.g. González-Morales et al., 2024) or over the course of an animal's ontogeny (e.g. Wilson, Heinsohn, & Endler, 2006). Yet, although such colour change may not always be primarily aimed at thermoregulation (colour frequently being used as social and sexual signal - e.g. Ligon & McGraw, 2013; camouflage - e.g. Wilson, Heinsohn, & Endler, 2006; etc.) it may often still have a thermal consequence (Walton & Bennet, 1993). Additionally, in some reptiles, structural features such as scale shapes, patterns or ridges, will have an effect on skin-surface properties such as roughness or reflectivity. This will, to some degree, inevitably modulate convective and radiant heat exchange processes (Lin et al., 2025), as well as rates of cutaneous evaporative water loss (EWL) (Sakich & Tattersall, 2021).

Ultimately, the effectiveness of reptilian thermoregulation arises from a complex integration of these behavioural, physiological and anatomical features. A desert lizard basking on a rock is not merely seeking warmth; it is engaging in a complex thermoregulatory process that involves microhabitat selection, postural optimization, and circulatory/physiological adjustments to achieve thermal homeostasis and optimal performance, all while considering a range of costs such as EWL rates, predation risk, and time not spent increasing fitness (eating, reproducing, social interactions, etc). Still, each strategy reinforces the others, creating a multifaceted response to environmental challenges that ultimately optimise the reptile's ability to navigate its environment.

As global temperatures rise, and habitats undergo rapid transformation, the resilience of reptile thermoregulation will face unprecedented challenges, but may also find new ecological opportunities. Understanding the interplay between behavioural, physiological, and anatomical strategies not only highlights the evolutionary ingenuity of these organisms but also provides a critical framework for predicting their responses to environmental change. The study of these integrative strategies reveals the depth of ectotherm adaptation, offering a glimpse into the delicate balance that sustains life in a thermally dynamic world.

1.1.3. Thermal Physiology Parameters, Performance Curves, Key Thermal Limits and Thermal Inertia

Understanding the relationship between body temperature and physiological function requires a framework that integrates thermal tolerance, preferences, and limits (Angilletta, 2009). Thermal performance curves (TPCs) and associated concepts such as thermal inertia, critical thermal limits, and the thermal set-point range provide critical insights into how reptiles interact with their thermal environment (Angilletta, 2009; Huey & Stevenson, 1979). These parameters not only explain physiological and behavioural responses of reptiles but also predict how species may respond to environmental changes, particularly under the anthropogenic pressures of habitat and climate change.

Preferred vs Optimal (vs Field) Body Temperatures

Reptiles usually exhibit some degree of thermal preference that aligns with their physiological needs, enabling them to optimize metabolic efficiency, locomotion and reproductive success. Thermal preference (also referred to as "preferred [body] temperature", or T_{pref}) refers to the temperature an animal seeks to maintain when actively and solely thermoregulating (i.e. while free from any other ecological constraints) (Dawson, 1975). This parameter is often taken as the mean (e.g. Gilbert & Miles, 2017) or median (e.g. DeWitt, 1967) (Camacho & Rusch, 2017) body temperature of an animal thermoregulating in a simple experimental set-up (e.g. thermal gradients - Light et al., 1966), ideally across the species' daily active period (Camacho & Rusch, 2017).

Thermal optimum (T_{opt}), on the other hand, represents the specific temperature at which performance peaks across a specific physiological function (Angilletta, Niewiarowski, & Navas, 2002) such as digestion, growth, locomotion as well as spermatogenesis/oogenesis and gestation. T_{opt} will also often be experimentally determined through establishing thermal performance curves (TPCs – discussed below). The two parameters (T_{pref} and T_{opt}) are intrinsically linked, as theorized in the thermal coadaptation hypothesis (Angilletta, Niewiarowski, & Navas, 2002), and even demonstrated in lacertid lizards (Bauwens et al., 1995). The hypothesis proposes that animals evolve to match their thermal preferences (T_{pref}) to the temperatures at which their physiological functions perform best (T_{opt}), ensuring that their thermoregulatory behaviour supports optimal biological performance in their native environment (Angilletta, Niewiarowski, & Navas, 2002). Hence, an animal will thermoregulate towards its T_{pref} in order to optimize the multiple physiological processes ongoing at any one point in time. Yet different physiological processes may have different T_{opt} (Angilletta, 2009;

Angilletta, Niewiarowski, & Navas, 2002) and hence T_{pref} will likely be a compromise between the T_{opt} of concurrent essential physiological processes, instead of precisely matching any specific one (Angilletta, Niewiarowski, & Navas, 2002). Considering this has major implications for defining T_{pref}, from both a conceptual and experimental point of view, as discussed further afield.

Laboratory studies have revealed remarkable behavioural precision in maintaining thermal preference (Sartorius et al., 2002). However, that is not to say that T_{pref} is always a fixed trait for an animal (Bowker et al., 2010), often being under the influence of aforementioned constraints such as predation pressure (e.g. Yuan et al., 2020) or reproductive/gestation status. For instance, gravid female lizards often shift their thermal preferences upward (e.g. in *Tropidurus spinulosus* - Juri, Chiaraviglio, & Cardozo, 2017) or downward (e.g. in lacertids - S'Khifa et al., 2023), to support embryonic development and/or to cope with the mother's higher demands of gestation. Ultimately, thermoregulation is often chaotic (Bowker et al., 2010 & 2013) and subject to circadian (and, in many cases, also circannual or seasonal) rhythms (Angilletta et al., 1999; Foà & Bertolucci, 2003; Karameta et al., 2023; Osojnik et al., 2013), further highlighting the dynamic nature of thermal preferenda based on life cycle and immediate physiological state or demands.

Yet, while T_{pref} and T_{opt} are experimentally determined, it is not uncommon for field studies to measure body temperatures of animals active in the field. Such temperatures, however, will often differ considerably from the experimentally attained aforementioned standards. This results from the fact that field thermal conditions, although livable, are rarely optimal (Taylor et al., 2020). Additionally, animals in the field are also subject to a range of other competing pressures (e.g. predators, hydration state, foraging, social interaction) (Angiletta, 2009; Camacho & Rusch, 2017; Taylor et al., 2020), otherwise absent from a controlled laboratory context where the sole concern becomes to thermoregulate optimally. Hence, another measure is often considered for these field contexts: the field-active body temperature (sometimes abbreviated to "field T_b"). This measure partially relates to the animal's thermoregulatory efforts and physiology, but it is also highly influenced by the animal's ecological context (e.g. thermal availability, competition, predation pressure, social context, etc.), immediate thermoregulatory history, and other extrinsic and intrinsic factors (e.g. water availability/ hydration state) (Rusch et al., 2017; Sannolo & Carretero, 2019). Ultimately, comparing these three parameters will yield relevant information regarding an animal's thermoregulatory strategies and ecological priorities.

Critical and Voluntary Thermal Limits & Other Thresholds

While T_{pref}, T_{opt} and field T_b represent the temperatures active animals normally seek to attain in order to optimize performance, and hence will likely be amongst the most frequent temperatures exhibited by the animals in their daily activities (Angiletta, 2009), critical and voluntary thermal limits, on the other hand, define the boundaries of an animal's thermal tolerance (Angilletta, Niewiarowski, & Navas, 2002; Lutterschmidt & Hutchison, 1997a; Taylor et al., 2020). Hence, these represent thresholds which the animals will actively avoid to reach or surpass (Taylor et al., 2020). Critical thermal limits (CT_{max} and CT_{min}) mark the upper and lower thresholds of temperature tolerance beyond which physiological function collapses (Angilletta, 2009; Angilletta, Niewiarowski & Navas, 2002, Huey, 1982). These are determined in laboratory settings by measuring widely accepted endpoints such as loss of motor control (Angilletta, 2009, Hertz, 1979) (e.g. loss of righting response - a commonly used standardized test - Angilletta, 2009 - yet one that may suffer from a behavioural bias as boldness may influence the response - Camacho & Rusch, 2017; Lutterschmidt & Hutchison, 1997a) or onset of muscle spasms (Lutterschmidt & Hutchison, 1997b). Although somewhat strict thresholds (especially CT_{max} – directly resulting from its strict biochemical underpinnings, discussed below and in Angilletta, 2009), these thresholds often provide insights as to the environmental context in which a given species has evolved (Angilletta, 2009).

The biochemical basis of these limits - reviewed in depth by Angilletta (2009) - provide insight into their rigidity and plasticity (Araújo et al, 2013; Bodensteiner et al., 2020. As covered in Angiletta's (2009) review, high temperatures, proteins (such as enzymes) denature and cellular membranes destabilize, thus markedly disrupting cellular homeostasis. These changes, especially protein/enzyme denaturation, are often irreversible and hence may lead to irrecuperable systemic failure and eventually death. Cold temperatures, on the other hand, lead to reduced biochemical and metabolic rates, enzyme inefficiency, and increased jellification of cell membranes (which, in turn, impairs cell structure and trans-membrane ion transport). Yet, although any of these conditions may still be lethal after a certain amount of time, in the short-term they are all nonetheless mostly reversible by reverting the temperature to some value above the CT_{min} threshold. This is unlike the irreversibility of processes, such protein/enzyme denaturation or cell membrane fusion, which happen at high temperatures, beyond CT_{max}. These biochemical underpinnings explain why CTmax tends to be stricter and more conserved across species than CT_{min} (Araújo et al., 2013; Grigg & Buckley, 2013; Herrando-Pérez et al., 2020a). Evolving an increased CT_{max} would require developing adaptations to higher temperatures. However, this is limited by the intrinsic, and often irreversible, thermal stability thresholds of biological macromolecules – namely proteins and

the phospholipids that form cell membranes – that form the basis of eukaryotic biochemistry. In contrast, CT_{min} exhibits greater evolutionary lability (Bodensteiner et al., 2020; Muñoz et al, 2014), reflecting the reversibility of cold-induced changes to cellular biochemistry, as well as the broader range of physiological, metabolic and behavioural strategies available to cope with the biochemical implications of cold temperatures. Still, as the interaction between thermo- and hydroregulation is increasingly investigated, studies have found some variability in these otherwise strict tolerance thresholds, depending on hydration state (Herrado-Pérez et al., 2020b), further emphasizing the long-ignored importance of water availability for reptile thermoregulation.

Ultimately, critical thermal limits represent the physiological boundaries beyond which life, for that organism, is not sustainable/viable. However, given the high stakes of reaching such temperatures, animals will normally abide by more conservative behavioural and physiological thresholds that fall well within such extremes (Taylor et al, 2020). Voluntary thermal limits (VT_{max} and VT_{min}), for instance, reflect behavioural thresholds, representing the temperatures at which reptiles seek to avoid adverse (yet still non-critical) thermal conditions (Camacho & Rusch, 2017; Camacho et al., 2018). These limits are ecologically relevant (Taylor et al., 2020), encompassing the temperature range reptiles must actively regulate within in order to avoid physiological thermal [heat/cold] stress.

Other behavioural thresholds exhibited by some reptiles include the temperatures at which animals begin to gape (gaping threshold, T_{gape}) and/or pant (panting threshold, T_{pant}) (Tattersall, Cadena, & Skinner, 2006; Taylor et al., 2020). These behavioural strategies enhance the rates of evaporative cooling from wet surfaces such as the mouth (gape and pant) and lungs (pant), promoting localised cooling of the head, and thus preventing the brain from reaching critical temperatures (e.g. Dorcas & Peterson, 1997; Sannolo et al., 2014; Tattersall, Cadena, & Skinner, 2006). They can also result in overall cooling of the animal, thus helping to avoid approaching CT_{max}, at a localised or systemic level (Loughran & Wolf, 2020 & 2023; Smith 1979). Hence, while for some species (e.g. many [terrestrial, mesic/xeric] lizards) these thresholds may be good indicators of systemic heat stress, representing emergency responses that incur an inevitable cost in hydration state (Loughran & Wolf, 2023; Plasman et al., 2025), in other more amphibious/tropical species it may be more of a strategy for localised cooling, reflecting the different thermal tolerance breadth of different organs and a decreased risk of dehydration (Loughran & Wolf, 2023; Plasman et al., 2025). Crocodilians, for instance, will often gape while continuing to bask (even with plentiful opportunity to shuttle to water in order to effectively and rapidly cool down) (Smith, 1975 & 1979; Spotila, Terpin, & Dodson, 1977). In such cases the animal is likely maximizing the heat acquired by its less

thermosensitive torso, while keeping the head region cooler by gaping, thus minimizing the potential for heat stress in the more thermosensitive brain.

The interplay between critical and voluntary limits provides important insights into reptile thermal ecology. While critical limits define physiological boundaries (i.e. the absolute thermal performance breadth = CT_{max} – CT_{min}), voluntary limits reflect the thermal breadth resulting from the behavioural strategies reptiles employ to avoid reaching thermal stress or said physiological boundaries (Camacho et al., 2018; Heatwole, & Firth, 1982). This distinction is particularly relevant in field studies, where voluntary limits often determine microhabitat selection and daily activity patterns (Camacho et al., 2018), while the imprint of critical limits may be more perceptible at a macroecological scale, such as delimiting a species' maximal potential range (Kingsolver, Diamond, & Buckley, 2013).

Ultimately, considering these thresholds of thermal limits and thermal tolerance, one can mechanistically infer how susceptible an animal may be to extirpation from a given environment with known thermal characteristics (Camacho et al., 2024, Huey, 1982) – e.g. the highest [air] temperatures observed in that environment. Furthermore, a parameter known as the "thermal safety margin", can be calculated as the difference between a species upper thermal tolerance threshold – typically CT_{max} , but VT_{max} or T_{pant} (Taylor et al., 2020) are also sometimes used instead – and the highest (or sometimes, mean) [usually air] temperature exhibited in the environment in question. In such cases, the higher the value of the thermal safety margin, the less risk the animal has of extirpation (Taylor et al., 2020). This parameter, albeit sparsely used, serves as a link between an animal's thermal physiology with its ecology and distribution.

Bridging Behaviour and Physiology: Thermal Set-Point Range and Other Thermoregulatory Indices

While a species' thermal physiology targets, such as T_{pref} and T_{opt}, may be well defined and experimentally reproducible, in practice the cost of consistently and precisely matching [field] T_b with T_{pref} (or any given T_{opt}) is often too great (see previous discussion on the costs of thermoregulation, as well as: Huey, 1982; Huey & Slatkin, 1976, Sears & Angilletta, 2015; Vickers, Manicom, & Schwarzkopf, 2011). Instead, an animal will usually aim to maintain its T_b somewhere within a range of temperatures which optimize physiological performance, and hence which will inevitably include T_{pref} as well as possibly even multiple T_{opt} (Angilletta, 2009; Huey, 1982). This is known as the thermal set-point range (T_{set}). It is usually taken to be the central 50% or 80% (Angilletta, 2009), depending on the study, most frequent body

temperatures chosen by an animal freely thermoregulating (without constraints) in a standardized experimental set-up such as a thermal gradient (Barber & Crawford, 1977; Light et al., 1966). Ultimately, this parameter serves as the bridge between behavioural thermoregulation and physiological targets (i.e. T_{pref} and T_{opt}) (Angilletta, 2009; Hertz, Huey & Stevenson, 1993), ensuring alignment between external actions and internal processes, while accounting for the animal's management of the inherent costs of precise thermoregulation.

Behavioural choices such as basking, shade-seeking, and substrate selection are intricately tied to maintaining the thermal set-point range (Huey, 1982). As for T_{pref}, with which it is intrinsically linked, T_{set} may also vary between species and/or populations of the same species, as well as across points in time (e.g. seasons) (Angilletta, 2009; Giacometti, et al., 2023). The variation in T_{set} can be represented by differences in the most commonly observed body temperature (i.e. a simple shift of the curve up or down the temperature axis - Fig. 1.3A), a difference in the breadth of the range of temperatures (i.e. a change in width of the curve - Fig. 1.3B), or both (Angiletta, 2009; Angilletta, Niewiarowski & Navas, 2002; Angilletta et al., 2003). While the first scenario illustrates adaptation to either cold or warm environments, the second scenario relates to differences in thermoregulatory precision associated with a [thermal] specialist (narrow T_{set}) vs generalist (broad T_{set}) trade-off (Angiletta, 2009; Angilletta, Niewiarowski & Navas, 2002; Angilletta et al., 2003; Levins, 1968), and the third scenario is a combination of the two. Of note, as illustrated in Fig. 1.3B), the TPCs of specialist-vs-generalist strategies depict a trade-off with maximal performance, often represented by the concept of "jack-of-all-temperatures, master of none" (Angilletta, 2009; Huey & Hertz, 1984; Taylor et al, 2020).

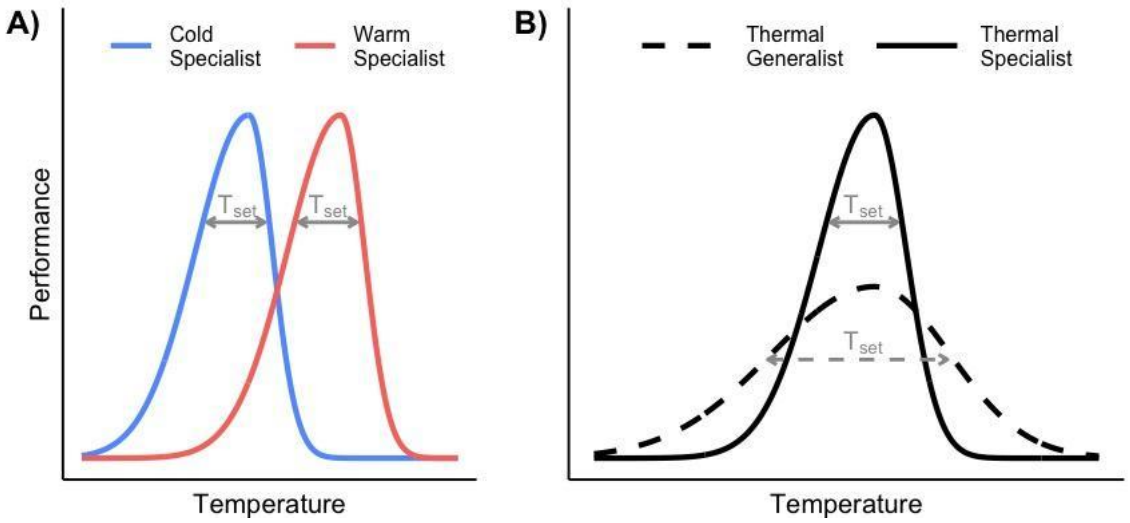

Fig. 1.3: Hypothetical TPCs for A) cold-adapted (blue line) vs warm-adapted (red line) organism, and B) a thermal specialist (full line) vs thermal generalist (dashed line) also showing the common trade-off between performance breadth and maximal performance. Note that in the hypothetical case in B) both species have the same T_{opt}, but this may not always be the case as each curve could be shifted left or right (depending on whether the species is more cold or warm adapted, respectively), independently of each other, as seen in A).

Additionally, the thermal set-point range may also vary throughout life stages (Bodensteiner et al., 2020; Hall & Warner, 2019; Lang, 1981). Juveniles, for instance, often have broader T_{set} ranges to accommodate rapid growth rates, to compensate for their heightened vulnerability, and potentially decreased access to prime thermoregulatory opportunities due to competition from adults and predation risk (Paulissen 1988). On the other hand, adult reptiles, particularly during the reproductive season, typically demonstrate tighter regulation, often in tandem with shifts in their T_{pref} (and T_{set}), reflecting the energetic and developmental demands of this critical period (Shine, 1980). Furthermore, dehydration and starvation have also been shown to affect T_{pref}, often flattening the performance curve, which reflect in changes to T_{set} (Brown & Griffin, 2003; Brown & Roberts, 2008; Sannolo & Carretero, 2019).

Hence, the concept of thermoregulatory precision relates to how an animal utilises behaviour and physiology to adjust the breadth of T_{set}, when given unconstrained (i.e. in a controlled laboratory context) thermoregulatory opportunities (Hertz, Huey, & Stevenson, 1993). However, in its original form (i.e. the range of T_{set}) this parameter fails to account for the skewness of the curve (Angilletta, 2006), hence it is often suggested that the area under the curve, within the thermal range itself, may be a better indicator of thermoregulatory precision.

Thus, two other concepts further describe how well an animal is able to thermoregulate in its natural environment. Firstly, the index of [absolute] thermoregulatory accuracy (d_b) is given by the absolute difference between field body temperature and the experimentally established set-point range ($d_b = | T_{set} - T_b |$) for that species (Angilletta, 2009; Christian & Weavers, 1996;

Hertz, Huey, & Stevenson, 1993). According to this index, the higher the d_b means the less accurate the animal is at thermoregulating (Angilletta, 2009; Taylor et al., 2020). Alternatively, by taking the simple (instead of absolute) difference between T_{set} and T_b, one can obtain the additional information regarding whether the animal is usually exhibiting T_b's above or below its T_{set}, providing an important context of whether an animal may be experiencing an increased risk of heat or cold stress, respectively (Taylor et al., 2020).

Secondly, indices are also used to relate how effectively an animal thermoregulates in an environment of a certain thermal quality (the latter given by $d_e = |T_{set} - T_e|$) (e.g. Christian & Weavers, 1996; Row & Blouin-Demers, 2006; Sartorius et al., 2002). Two approaches have been used to establish thermoregulatory effectiveness as a relationship between an animal's thermoregulatory accuracy (d_b – see above) and the environment's thermal quality (d_e): Hertz et al. (1993) proposed calculating thermoregulatory effectiveness (E) as E = mean d_b / mean d_e , while Blouin-Demers & Weatherhead (2002) later propose a simpler relationship whereby thermoregulatory effectiveness (I) is given by $I = d_e - d_b$. Regardless, both approaches are able to distinguish whether an animal is thermoregulating carefully, thermoconforming or even if they are entirely avoiding thermally favourable habitats (i.e. thermoregulatory avoidance - in itself a form of [negative] thermoregulation). However, these approaches are inevitably plagued by the fact that, by using operative temperatures (T_e) as an estimate of potentially available body temperatures to the animal, one is assuming zero heat capacity of the subject (Christian & Weavers, 1996, Seebacher & Shine, 2004). While this may be an acceptable assumption for smaller animals, it is not for animals with larger mass, which exhibit significant thermal inertia, emphasizing the need to apply correction factors that account for body mass (Seebacher & Shine, 2004).

Thermal Performance Curves: Linking Body Temperature to Function

While the aforementioned indices and thresholds shed some light as to how and to what extent an animal attains or prefers a certain body temperature (or range of temperatures), they do not illustrate the implications of body temperature(s) on an animal's physiological performance. To achieve this, thermal performance curves (TPCs) must be established (Angilletta, 2006 & 2009; Angilletta, Niewiarowski, & Navas, 2002; Huey, 1982). By illustrating the relationship between T_b and performance, TPCs also provide a predictive framework for understanding reptilian function across diverse thermal environments (Huey & Stevenson, 1979). These curves are often bell-shaped, modelled with a skewed Gaussian function (Angilletta, 2006), with performance increasing to an optimal point (T_{opt}) before declining

sharply at extreme temperatures (Fig. 1.4). Furthermore, the general skewed bell-shape of TPCs - with a steeper decline in performance at temperatures beyond T_{opt} than the slope of the increase in performance with temperatures leading towards T_{opt} - is very conserved across traits and taxa (Arnoldi et al, 2025). Nevertheless, depending on the species and/or trait being assessed, the typical bell-shaped curve may often be increasingly warped or asymmetrical (Bulté & Blouin-Demers, 2006). Hence functions or distributions other than Gaussian, have also been used to model TPCs. These have included general additive models, polynomial functions, β-distribution and other functions - as discussed by Angilletta (2006).

The breadth and shape of a TPC reflect a species' thermal adaptability and evolutionary history (Angilletta, Niewiarowski, & Navas, 2002). Moreover, under the thermal coadaptation hypothesis (Angilletta, 2009; Angilletta et al., 2003; Angilletta, Niewiarowski, & Navas, 2002), TPC's and thermal optima are expected to align with the environmental temperatures typically experienced by the species, reflecting evolutionary adaptation to local thermal conditions (see also "*Bridging Behaviour and Physiology: Thermal Set-Point Range and Other Thermoregulatory Indices"*). Hence, TPCs are valuable tools for interpreting an animal's thermal ecology. For instance, in generalists, the curve is broader, encompassing a wide range of temperatures over which performance is maintained (Angilletta, 2009; Huey & Hertz, 1984), albeit at suboptimal levels (Fig. 1.3B). This flexibility enables thermal generalists to thrive in variable environments. In contrast, specialists display narrower curves, with steep declines in performance outside their T_{opt} (Fig. 1.3B). This specialization allows for peak performance in stable habitats but limits their adaptability to environmental fluctuations (Angilleta, 2009; Huey & Hertz, 1984). Furthermore, the trade-offs associated with thermal specialization and generalization can also be interpreted from the shape of TPCs (Fig. 1.3B). In theory, specialists achieve higher peak performance in narrow thermal niches (i.e. taller but narrower TPC) while generalists sacrifice peak performance for broader tolerances (i.e. shorter but broader TPC) (Angilletta, 2009). In practice, this difference has important implications in terms of resilience to habitat or environmental/climate change, with generalists potentially having the "upper hand" in the face of changing conditions, given their broader thermal tolerance (Angilletta, 2009). Nevertheless, empirical support for this claim is still, to a degree, scarce and, at times, contradictory (e.g. Ortega, Mencía, & Pérez-Mellado, 2016), as several other factors (namely the magnitude of the *thermal safety margin*) beyond simply the breadth of T_{set} play a part in determining the cost of thermoregulating in a novel/changed environment (Angilletta, 2009; Sinclair et al., 2016).

This introduces yet another important concept that also arises from the shape of the TPCs which is the *performance breadth* ($\%T_{br}$). Despite variations in its definition, performance

breadth is usually taken as the range of temperatures at which an animal performs above a given [maximal] level (Taylor et at., 2020). A commonly used threshold is 80% (i.e. 80%T_{br} - temperature range where 80% of maximal performance is observed) (Taylor et al., 2020), but other thresholds can also be used in the literature (e.g. 95%T_{br}, or even the 100%T_{br} – which represents the absolute thermal performance breadth, but which is sometimes simply abbreviated to *performance breadth*), depending on the study in question (Angilletta, 2009; Bodensteiner et al., 2020). Other key thermal physiology parameters also fit into these curves (Fig. 1.4). T_{set} (and T_{pref}) represents the range (or point, respectively) an animal prefers to actively regulate its body temperature towards, and typically aligns closely with the steepest upward slope of the TPC. T_{opt}, where performance peaks (i.e. the tip of the TPC), often lies around the midpoint of T_{set}. CT_{max} and CT_{min} define the upper and lower boundaries of the curve, marking the temperatures beyond which performance collapses (Angilletta, 2009; Camacho & Rusch, 2017; Huey, 1982; Huey & Stevenson, 1979).

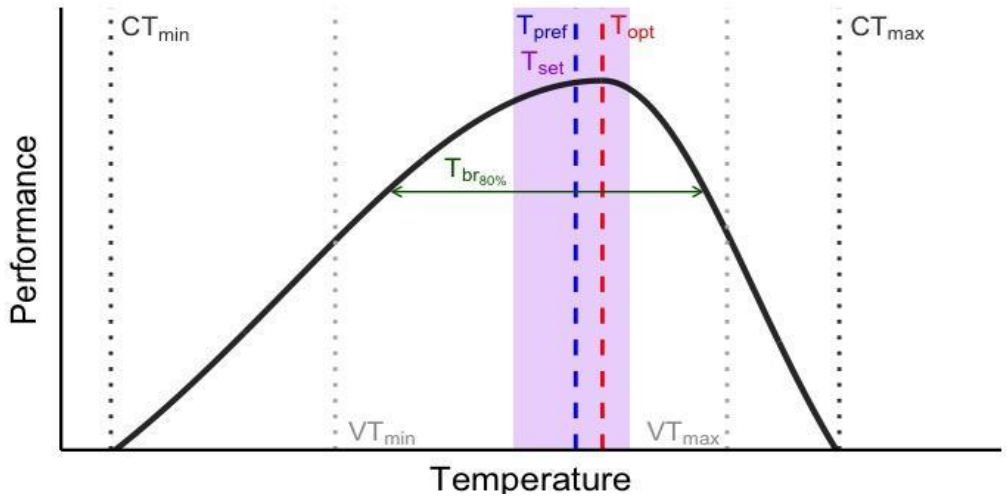

Fig. 1.4: Typical shape of a Thermal Performance Curve (TPC) for a hypothetical thermal-sensitive trait (e.g. maximum speed) showing annotations of the several thresholds discussed prior and demonstrating how they fit within the shape of the TPC. Note the typically asymmetrical (left skew) shape, characteristic of most TPCs.

Thermal Inertia: The Role of Body Size and Shape in Heat Transfer and Distribution

Conceptually, thermal inertia describes a body's inherent ability to resist rapid changes in temperature as a consequence of its mass and thermal conductance (Bell, 1980). Hence, it is a property that will inevitably affect the thermoregulatory effort of any animal. Nevertheless the magnitude of its effect, and hence its contribution towards an animal's thermoregulation, is highly dependent on the size (i.e. mass) of said animal. For instance, larger reptiles, such as crocodilians, monitor lizards and many chelonians, exhibit significant thermal inertia due to their greater thermal mass, which significantly slows heat gain and loss (Don & Brown, 2017; Paladino, 1990). Similarly, estuarine crocodiles (*Crocodylus porosus*), for example, can maintain stable body temperatures over prolonged periods (Grigg et al., 1998), allowing them to exploit thermally variable environments (see "*Thermoregulatory Strategies in Reptiles: Behaviour, Physiology, and Anatomy*" above).

The effect of thermal inertia is governed by the interplay of mass, shape, and surface area-to-volume ratio (Bell, 1980). Heat exchange happens mostly (although not exclusively - e.g. Tattersall, Cadena, & Skinner, 2006) across the external surfaces of the animal (Porter & Gates, 1969; Porter, et al., 1973). However, while an animal's surface area will approximately scale with the square of an animal's length, its volume (and hence, its mass) will follow a cubic relationship with an animal's length (Okie, 2013). As a result, larger bodies will typically possess a comparatively lower surface area-to-volume ratios, aiding to reduce the rate of heat exchange with the environment (Bell, 1980). Furthermore, a large body size also typically means a larger distance between the animal's surface and its core, and between different body parts. This will further emphasize any surface-to-core temperature gradients (Porter & Gates, 1969) and patterns of regional heterothermy respectively. Hence, while the surface of a large animal may experience large thermal fluctuations, the large amounts of tissue (i.e. mass) will buffer such surface fluctuations from reaching the animal's core, ultimately contributing towards the internal thermal homeostasis observed in inertial homeotherms (McNab, 1978).

Additionally, the reptilian body plan shows remarkable diversity, encompassing animals with markedly different shapes (Brocklehurst, Ford & Benson, 2022). Such variation in shapes will inevitably impact surface-area-to-volume ratios and hence the effect of thermal inertia on an animal's body temperature (Garrick, 2008). Compact and rotund shapes, such as those of many chelonians as well as of "bulkier" lizards further decrease this surface area-to-volume ratio, thus enhancing thermal buffering (Zamora-Camacho, Reguera & Moreno-Rueda, 2014). Contrastingly, elongate shapes, such as those of snakes and many skinks, will have the contrary effect on this ratio (Cox et al., 2023). Nevertheless, some elongate species will

behaviourally compensate for their increased surface area-to volume ratio by adopting a coiled position which simulates a more compact body plan and reduces thermal loss (Heatwole & Johnson, 2008).

The thermal time constant (τ) - a measure of the time it takes for an organism to reach thermal equilibrium with its environment (Dzialowski & O'Connor, 2001a) - is directly influenced by the aforementioned physical properties of the animal as well as environmental factors (Bakken & Gates, 1975). For instance, at any given set of environmental parameters, larger reptiles (i.e. with high thermal inertia) have longer thermal time constants, enabling them to maintain stable body temperatures but requiring extended basking periods to achieve T_{opt} (Turner & Tracy, 1985). Conversely, smaller reptiles, such as many geckos and skinks, lack substantial thermal inertia (i.e. small τ), exposing them to rapid temperature fluctuations (Bell, 1980; Claunch et al., 2020; Fraser & Grigg, 1984). While this enables quick adjustments and fast heat acquisition, it also increases their vulnerability to thermal stress in unstable environments (Giacometti et al., 2023). Hence, smaller animals will rely heavily on behavioural thermoregulation to compensate for their low inertia, carefully adjusting body orientation in regards to solar radiation or shuttling frequently between microhabitats to avoid overheating (Giacometti et al., 2023; Black & Tattersall, 2017; Bohórquez-Alonso et al., 2011).

Similarly, while whole-animal thermal inertia will affect an animal's thermal exchange rate and hence its overall body temperature, at a finer scale, the different sizes, shapes and surface area-to-volume ratios of an animal's body parts will result in localised differences in thermal inertia (Claunch et al., 2020). Hence, these local differences may result in different localised thermal exchange rates, thus contributing towards consolidating patterns of regional heterothermy (Heath, 1964). Furthermore, animals (especially larger ones, such as crocodilians and large chelonians) can capitalise on these differences in thermal inertia across the body to optimise heat storage and subsequent redistribution (i.e. source-sink dynamics) over extended periods of time (Grigg & Alchin, 1976). For example, a large crocodile may utilise the large thermal mass of its torso to accumulate heat during a sunny day, to then redistribute this heat to essential organs and appendages (e.g. brain, tail) during night-time activities, effectively increasing its active period of optimal performance.

Ultimately, thermal inertia and thermal time constants are often overlooked parameters of an animal's thermoregulatory toolkit. They have important ecological implications regarding how a species mediates interactions with its environment. Furthermore, they also raise relevant theoretical and methodological considerations that must be taken into account when measuring an animal's body temperature(s) and studying its thermal ecophysiology.

1.2. Historical and Conceptual Foundations of Reptile Thermal Ecology Studies

1.2.1 Early Descriptive Studies and Foundational Observations

The first attempts to measure reptile body temperatures in the field were rudimentary, relying on simple thermometers and manual data collection (Bogert, 1949). For example, in the early 20th century, mercury thermometers were inserted into the cloacae of captured reptiles to estimate core temperatures (Brooks, 1968). While labour-intensive, these measurements provided invaluable insights into how body temperatures deviated from ambient conditions and provided the first evidence for active thermoregulation in reptiles (e.g. Mueller, 1969). These findings spurred a booming interest in understanding the relationships between body temperature, physiology and ecological success, as reptiles were revealed to thrive in environments where extreme thermal conditions might incapacitate other organisms.

An important milestone in reptile thermal ecology came in the 1960s with the pioneering work of John Heath. Through his ground-breaking work on horned lizards (Heath, 1964), Heath demonstrated that body temperature in lizards was not uniform across their body. Instead, it varied significantly across regions thus providing the first empirical evidence of regional heterothermy in small reptiles. He observed, for instance, that during basking, the head and anterior regions of a lizard often warmed more rapidly than the rest of the body. This has since been hypothesized as an adaptation prioritizing optimal thermal conditions for neural and sensory functions (Sannolo et al., 2014), critical for an animal's survival. These findings not only challenged the prevailing assumption of uniform body temperature but also highlighted the adaptive value of regional heterothermy in optimizing physiological performance of specific organs or regions of the body (Webb, Johnson, & Firth, 1972).

Heath's work also underscored the methodological implications of regional heterothermy for measuring and interpreting body temperatures. In itself, it challenged the idea of a single body temperature being representative of general internal thermal state. While cloacal temperatures were, and still are, commonly used as a proxy for core thermal state, the presence of regional heterothermy means this single point may not capture the spatial variability observed in surface or regional temperatures (e.g. Barroso et al., 2016). The complexity of the observed patterns of regional heterothermy, coupled with an increased awareness of the presence of surface-to-core temperature gradients and of the interactive effects of thermal inertia, highlighted the need for a more nuanced approach to obtain and interpret body temperature measurements. This heavily influenced subsequent studies, which gradually started to

incorporate multiple measurement sites and advanced technologies capable of capturing thermal gradients across the body of the animal (Taylor et al., 2020).

Other early, and mostly descriptive, studies focused widely on the interplay between behaviour and body temperature. In the field of crocodilian thermal ecology, for example, these early studies provided critical insights into the thermoregulatory behaviours of these semi-aquatic reptiles. Observations on estuarine crocodiles (*Crocodylus porosus*) documented their use of basking to elevate body temperatures and their tendency to submerge in water to avoid overheating, coupling both behavioural observations with body temperature readings (Barham et al., 2024; Grigg et al., 1998). These behaviours highlighted the importance of both terrestrial and aquatic habitats in maintaining thermal balance. Similarly, studies on freshwater crocodiles (*Crocodylus johnstoni*) revealed diel patterns of thermoregulation, with individuals transitioning between basking and aquatic cooling to navigate the thermal heterogeneity of their environments (Johnson, Webb, & Tanner, 1976; Nordberg & McKnight, 2023; Seebacher, 1999). Concurrently, similar studies further reiterated the importance of behavioural strategies - many of them quite ubiquitous amongst reptiles in general (e.g. basking) - across all reptile groups (Narayanan, 2025; Smith, 1979). Nevertheless, perhaps due to logistical convenience (e.g. small size, ease of handling/capture, accessibility, etc.), many such studies focused on small or medium sized lizards.

A notable characteristic of these early studies, though, was their correlative nature, with many studies resorting to comparing animal body temperatures to environmental variables such as air temperature (Brattstrom, 1965), solar radiation (Bogert, 1949), and substrate temperatures (Brattstrom, 1965). However, the correlative nature of these seminal studies offered both strengths and limitations. On the one hand, correlational studies required minimal intervention, mostly preserving the natural behaviour of animals and allowing the identification of key environmental variables influencing thermoregulation (Rozen-Rechels et al., 2019). Important conclusions were drawn from such studies, for example, comparisons of habitat use by desert and tropical reptiles revealed how thermal environments can shape species distributions, behaviours and broad thermoregulatory strategies (Grimm-Seyfarth, Mihoub & Henle, 2017; Narayanan, 2025). However, this approach often lacked the precision needed to disentangle complex interactions between physiology, behaviour, and environmental conditions. Without controlled experimental designs, these studies struggled to infer causality (Taylor et al., 2020), leaving critical questions regarding the mechanisms underlying thermoregulation unanswered.

The limitations of early correlative studies prompted an increased interest in more experimental and mechanistic approaches (e.g. Peterson, Papeş & Soberón, 2015).

Researchers began designing controlled experiments to manipulate environmental variables, such as light intensity or substrate temperature, and measure their effects on body temperature, behaviour, and physiological performance. These mechanistic studies, though logistically more challenging than their correlative counterparts, offered greater resolution and predictive power (Taylor et al., 2020). For instance, experimental studies using artificial thermal gradients (Light et al., 1966) or, less commonly, choice chambers (Camacho & Rusch, 2017), employed controlled and repeatable methods to identify preferred temperature ranges (T_{pref}) and set-point (T_{set}) regulation, allowing for evidence-based conclusions to be drawn on the active thermoregulatory behaviours and underlying thermal physiology of reptiles (Taylor et al., 2020).

The transition from correlative to mechanistic studies also brought into focus the need for more integrative methodologies. In crocodilian research, for example, the combination of field observations with experimental setups revealed how behavioural thermoregulation intersected with physiological processes such as metabolic rate and oxygen consumption (Johnson, Webb, & Tanner, 1976). Early investigations into crocodilian thermal inertia demonstrated the role of body size in buffering temperature fluctuations, providing a mechanistic explanation for their ability to exploit thermally variable habitats (Grigg et al., 1998).

Despite the advancements they have driven, mechanistic approaches are not without challenges. Laboratory experiments, while controlled and repeatable, often fail to replicate the complexity of natural habitats, including microclimatic variability, predation risk, and social interactions (Kearney et al., 2018; Wu et al., 2025). These discrepancies underscore the importance of integrating field and experimental data to achieve ecological relevance. For example, while laboratory studies might establish the thermal preferences of a reptile under stable conditions, field observations reveal how these preferences are mediated by behavioural and ecological trade-offs, such as the need to avoid predators, find food or a mate, avoid dehydration, or compete for basking sites (Sannolo & Carretero, 2019; Sannolo et al., 2019; Žagar et al., 2015).

The recognition of gaps in earlier methods motivated a call for more detailed and integrative approaches, laying the groundwork for subsequent advancements in the field. By emphasizing the need for improved tools, repeatable protocols, and a mechanistic understanding of thermal exchange processes, researchers began to address the complexities of reptilian thermoregulation more effectively. An example of this, the recent emergence of studies resorting to mesocosm systems that attempt to mimic natural environmental conditions in a controlled and replicable but small/ manageable space (Perry et al., 2025; Simbula & Barroso

et al, 2025). These aim to facilitate undertaking of replicable common-garden experiments that seek to balance the ecological representativeness of field studies, with the control and repeatability of laboratory experiments.

Despite the wider array of tools and methods now employed to study reptile thermal ecophysiology, the early descriptive and correlational studies, though constrained by technology, were instrumental in establishing the foundational patterns of thermal diversity and adaptation in reptiles, setting the stage for the high-resolution models and tools that define the field today.

1.2.2. Thermal Exchange Rates, Thermal Inertia, and Conceptual Refinements

Another major historically relevant question on the field of reptile thermal ecology revolved around the challenge of understanding how reptiles, and other ecotherms, exchange heat with their environment. Early studies sought to approximate thermal exchange rates and infer their ecological implications, relying on simple tools and basic - often purely theoretical - conceptual frameworks (e.g. Pough, 1980; Stevenson, 1985). These efforts, while ground-breaking for their time, further revealed the complexity of reptile thermoregulation and highlighted significant gaps that would eventually drive the field toward more mechanistic and integrative approaches, as described prior.

One of the earliest tools for studying thermal exchange was the use of copper models designed to mimic the thermal properties of reptiles in various habitats (e.g. Bakken, 1989). These models, often cylindrical (Vickers & Schwarzkopf, 2016) or shaped to approximate lizard body structure or silhouette (Shine & Kearney, 2001), were deployed in natural settings to measure rates of heat gain and loss under different environmental conditions. By simulating the effects of radiation, convection, and conduction on a thermoconforming body, copper models offered an accessible means to quantify thermal environments (i.e. instrumental for establishing operative temperatures – T_e) and compare them to reptile behaviour and field body temperatures (Bakken & Angilletta, 2013). For instance, studies used these models to demonstrate how desert lizards avoided extreme midday heat by seeking shaded refuges while optimizing basking opportunities during cooler parts of the day (Grimm-Seyfarth, Mihoub & Henle, 2017; Stark et al., 2024).

Although copper models provided valuable insights, their limitations became apparent as researchers sought to generalize findings across species and habitats. The models lacked the ability to account for biological variability, such as differences in body size (and hence, in

inertia), shape, or surface properties, which could significantly alter thermal exchange rates (Lutterschmidt & Reinert, 2011; Seebacher & Shine, 2004). These discrepancies raised questions regarding the ecological relevance of early operative temperature estimates – still a divisive topic amongst reptile thermal ecologists - and emphasized the need for more biologically accurate approaches (e.g. Seebacher & Shine, 2004). Additionally, the use of copper models highlighted the absence of dynamic feedback mechanisms, such as the behavioural and physiological adjustments reptiles employ to modulate heat exchange in real time (Seebacher & Franklin, 2005). These gaps laid the groundwork for later innovations that would integrate physiological, anatomical, and behavioural perspectives into thermal studies. For instance, observations of desert species, such as *Dipsosaurus dorsalis*, showed that preferred microhabitats often aligned with thermal preferences, while individuals actively avoided extreme temperatures through shuttling behaviour (Berk & Heath, 1975).

Thermal inertia further complicated early conceptual models. The observation that larger reptiles, such as monitor lizards and crocodilians, maintained more stable body temperatures compared to smaller species introduced the concept of thermal inertia as a critical factor in thermoregulation. Researchers recognized that body size, shape, and mass influenced heat retention and thermal exchange rates, but early methods struggled to quantify these effects accurately (Claunch et al., 2020). Copper models and passive sensors could approximate environmental conditions but were unable to account for the thermal buffering capacity conferred by body mass and surface-to-volume ratios. These limitations prompted questions about how thermal inertia interacted with physiology and behavioural strategies, such as prolonged basking or shade-seeking, to bring the animal closer to thermal stability (Seebacher & Shine, 2004).

The gaps in early methodologies also extended to the definition and measurement of key thermal parameters, such as thermal preferences and critical limits. While thermal gradients in laboratory settings provided a controlled environment for assessing these parameters, their relevance to natural habitats remained a subject of debate. Field studies revealed that environmental conditions often imposed constraints on thermal preferences, forcing reptiles to operate outside their preferred or optimal ranges (Vujović, Pešić and Meek, 2025). These discrepancies between laboratory findings and field observations highlighted the need for standardization in experimental protocols and for methods that could bridge the gap between controlled and natural settings (Camacho & Rusch, 2017; Faye et al., 2016; Taylor et al., 2020).

By the late 20th century, it was clear that existing tools and methods were insufficient to capture the full complexity of reptile thermal ecology. The reliance on static measurements

and simplistic models prompted critical questions regarding the interplay between thermal exchange rates, behaviour, and physiology. How did thermal inertia influence daily and seasonal activity patterns? To what extent did behavioural strategies compensate for environmental variability, and at what cost? What methods could more accurately reflect the dynamic nature of thermoregulation in reptiles? These unanswered questions became the driving force behind the development of high-resolution tools and integrative approaches, which would later transform the field.

The historical focus on thermal exchange rates and the limitations of early methodologies underscored the need for conceptual refinements and methodological advancements. By exposing gaps in understanding and raising critical questions, these early studies laid the foundation for the advanced mechanistic and predictive models that would emerge in subsequent decades. The lessons learned from these foundational efforts continue to inform modern approaches, ensuring that the study of reptile thermal ecology remains grounded in a deep appreciation for the complexity of thermodynamic principles of heat exchange and of the multidimensional nature of thermoregulatory strategies.

1.2.3. Operative Temperatures, Thermal Niches, and Emergence of Mechanistic Models

The conceptual leap from correlational approaches to mechanistic understanding marked a pivotal era in reptile thermal ecology. Recognizing that body temperature alone could not fully explain the dynamic interplay between reptiles and their environments, researchers began to look at operative temperatures (T_e) **-** a measure of the thermal environment as experienced by an organism – under a different light. This shift refined the study of thermal niches and laid the groundwork for mechanistic models that linked thermal preferenda and several other thermo-physiological thresholds to habitat use and species distributions (Frishkoff, Hadly & Daily, 2015; Kearney et al., 2018).

Operative temperature models addressed key limitations of earlier methods that relied on correlating body temperature with various environmental variables. While useful for identifying broad patterns, the latter correlational approaches often failed to capture the complexity of heat exchange processes or the thermal heterogeneity of habitats (Taylor et al., 2020). Operative temperature models bridged this gap by simulating the thermal properties of reptiles within their environments. By measuring heat gain and loss under varying conditions, these models offered a more nuanced representation of the thermal landscapes reptiles navigate (Fei et al, 2011).

Earlier physical models, such as copper cylinders, recently gave way to more anatomically accurate - even 3D-printed - animal replicas (Alujević et al., 2023; Shine & Kearney, 2001), which have been key tools in this transition. Designed to mimic the thermal responses of live, non-thermoregulating animals, these models quantified how environmental factors like solar radiation, wind, and substrate temperature influenced the realised thermal availability. Furthermore, increased complexity of these physical models allowed studies to explore a range of other variables, such as body postures (Bakken & Angilletta, 2013), inertial effects (e.g. through water-filled models - Lutterschmidt & Reinert, 2012), coloration (e.g. through painted models - Snyder, Tracy & Nussear, 2019), etc, and how they impact thermoregulation.

Integrating operative temperatures with thermal preferences and critical limits advanced the concept of thermal niches. Early ecological theories, such as Hutchinson's niche framework (Soberón & Arroyo-Peña, 2017), described the environmental conditions a species could tolerate but lacked detailed incorporation of physiological performance and other functional traits. Operative temperature models allowed researchers to quantify the thermal environments available to reptiles and compare these conditions to laboratory-determined parameters, such as T_{pref}, CT_{max} and CT_{min}, as well as VT_{max} and VT_{min} (Angilletta, 2009). This integration transformed niche concepts into predictive tools for understanding how thermal constraints shaped habitat selection (Vasconcelos, Santos & Carretero, 2012), activity patterns (Gunderson & Leal, 2015), and range distributions (Kearney & Porter, 2009).

For instance, studies on *Sceloporus* lizards revealed that individuals often operated outside their preferred thermal ranges due to habitat constraints (Plasman et al., 2024). These findings underscored the importance of behavioural flexibility (e.g. basking, shade-seeking, and timing of activity) in mediating the mismatch between environmental conditions and physiological optima. By linking operative temperatures to critical limits and behavioural adaptations, studies refined predictions relating to habitat use, activity budgets and resilience in response to environmental variability, providing further support for the continued use of operative temperature models as a null model of thermal availability.

The emergence of mechanistic models further enriched thermal niche research by incorporating dynamic factors, such as metabolic rates and water loss, into predictive frameworks (Kearney et al., 2012). These models also advanced the study of species distributions by integrating thermal niches with environmental data and other biological functions such as hydroregulation, feeding and reproduction into a common thermodynamic framework (Briscoe et al., 2023; Kearney et al., 2012). By quantifying the thermal resources available in a habitat and comparing them to species-specific physiological limits and requirements, studies have predicted range boundaries and assessed vulnerability of species

to climate change (Camacho et al., 2024). For instance, models predicting shifts in the distribution of *Anolis* lizards under warming scenarios demonstrated how limited thermal niches exacerbated risks for some species with narrow tolerances (Neel et al., 2021). These findings highlight the importance of thermal ecology in informing conservation strategies for reptiles facing rapidly changing environments (Muñoz et al., 2021).

The transition from correlational to mechanistic approaches redefined the study of reptilian thermal ecology, elevating it from a descriptive discipline to a predictive science. By integrating behaviour, physiology, and environmental heterogeneity, operative temperature models and mechanistic frameworks offered a deeper understanding of how reptiles navigate their thermal landscapes. These advancements not only refined ecological theory but also provided essential tools for addressing the challenges posed by habitat loss and climate change, ensuring that the legacy of early research continues to inform the field's evolution.

1.3. Evolution of Methodologies and Modern Tools in Thermal Ecology Studies

1.3.1. Measuring Body Temperatures and Determining Key Thermal Parameters

The accurate measurement of body temperatures is foundational to understanding the thermal ecology and physiology of reptiles. This data is essential for determining key thermal parameters, such as those discussed prior, which together define how reptiles interact with their environment. Advances in technology have introduced sophisticated tools and methods for temperature measurement, yet traditional techniques remain widely used due to their reliability, accessibility and comparability to past data and well-established thresholds.

Traditionally, cloacal thermometers have been the gold standard for measuring body temperatures in reptiles (Angilletta, 2009; McMaster & Downs, 2013; Taylor et al., 2020). By inserting a probe into the animal's cloaca, one can obtain an estimate of its internal thermal state. This method has several advantages: it is simple, usually cheap, widely applicable across species, and provides a direct measure of the thermal conditions experienced by critical physiological systems around an animal's core (Angilletta, 2009). However, it requires handling the animal, which can, in itself, influence the results in several ways, including by transferring heat from the researcher (Langkinde & Shine, 2006), and inducing stress (Bucklin, 2006) which can have a direct effect in its physiology and behaviour (Sannolo et al., 2014). These effects can be particularly serious for small or cryptic species. Moreover, the method assumes that the cloacal temperature accurately represents the animal's overall thermal state,

which, as discussed earlier, will not always hold true for species exhibiting regional heterothermy (e.g. McMaster & Downs, 2013) or during periods of rapid temperature change (Cox et al., 2023), particularly in large animals – who will have a large thermal inertia and significant regional heterothermy.

To address these limitations, alternative approaches have been developed. Temperature-sensitive probes can be inserted into other body regions, such as the oesophagus or muscle tissue (e.g. Bassetti et al., 2020; Cremer et al., 2019), to capture anatomically-specific temperatures. These methods provide a higher-resolution understanding of thermal physiology, particularly for studies focused on localized processes like digestion or muscle performance. However, they may require surgical implantation or other invasive procedures, limiting their application in certain contexts and inevitably impacting an animal's behaviour. More recently, external attachments such as diminutive loggers (e.g. Perry et al., 2025; Vickers & Schwarzkopf, 2016; and many more) and adhesive sensors (e.g. Nordberg & Schwarzkopf, 2019) have been employed to continuously monitor skin surface temperature changes in field or semi-natural settings (e.g. mesocosms) settings (Reyes-Puig et al., 2025; Perry et al., 2025), offering insights into diel thermal patterns without requiring repeated handling. Nevertheless, care must be taken when extrapolating skin-surface temperatures to core ones, as the effects of surface-to-core gradients, thermal inertia and regional heterothermy must be carefully accounted for before inferring internal state from external measures (Barroso et al, 2016).

As also discussed prior, regional heterothermy, where different parts of the body maintain distinct temperatures, introduces complexity to the measurement of body temperatures and the determination of thermal parameters. For example, in some snakes, the head and neck regions have been shown to retain higher temperatures than the cloaca or body core during basking, in order to maximize neural and sensory function (Cox et al., 2023 & 2024). The opposite pattern, however, has been observed in many lizards, where cephalic temperature is often lower than general core proxies (Webb, Johnson & Firth, 1972), potentially as a mechanism to protect the brain from the risk of overheating (Sannolo et al., 2014; Tattersall, Cadena, & Skinner, 2006). Ultimately, this phenomenon raises the important question of which temperature (core, surface, or regional; and if so, then head, skin, or cloacal), if any one in particular, should be used as a standard for assessing thermal preferences and physiological targets. While cloacal temperature is often considered a reliable proxy for core thermal state (Angilletta, 2009; Taylor et al., 2020), surface temperature measurements may provide valuable context for understanding heat exchange processes (Clarac et al., 2017; Garrick, 2008). Hence, arguably, the choice of measurement site should therefore align with the

specific goals of the study and the species being examined. One important consideration, however, lies in the fact that historically, most body temperature thresholds (e.g. T_{pref}) have been defined based on cloacal temperature measurements (Angilletta, 2009; Camacho & Rusch, 2017). Hence, while this measure may not always represent the most complete or even accurate portrayal of "general" internal state, the need for retrospective comparability with previously published thresholds or historical studies, may (in the absence or accurate calibrations) override the benefits of increased accuracy from other measures of core state.

Standardized laboratory methods, such as thermal gradients and choice chambers, have long been used to determine thermal preferences and voluntary thresholds in reptiles (Agilletta, 2009; Camacho & Rusch, 2017; Clusela-Trullas & Chow, 2013; Light, Shoemaker, & Main, 1966; Taylor et al., 2020). These experimental setups allow animals to move between zones of varying temperatures, enabling researchers to observe their thermal selection behaviour, and measure their respective body temperature, under controlled, simplified conditions (e.g. Laterza-Barbosa et al., 2015). For instance, reptiles placed in a thermal gradient will typically select body temperatures within their preferred temperature (T_{pref}) or set point range (T_{set}) (Angilletta, 2009; Taylor et al., 2020), providing direct insight into their set-point regulation. Similarly well-established experimental methods are equally useful for determining critical and voluntary thermal maxima (CT_{max} and VT_{max}) and minima (CT_{min} and VT_{min}) through controlled heating or cooling protocols that identify the points at which physiological function ceases or active behavioural avoidance occurs, respectively (Camacho et al., 2018; Roseno et al., 2024; Camacho & Rusch, 2017). These data are invaluable for comparative studies across species and populations, as they provide a consistent framework for evaluating and comparing thermal tolerance and adaptability. Still, despite their proven replicability and usefulness, these methods are inevitably plagued by often depending on a behavioural response (e.g. righting response for CT_x; moving to/from shelter for VT_x), which is in itself somewhat dependent on the boldness/shyness of the subject thus potentially introducing biases into the results (Diele-Viegas et al., 2018; Kashon & Carlson, 2018).

Furthermore, while laboratory settings usually offer precision and repeatability, they also present challenges (Clusella-Trullas, Terblanche et al., 2007). The artificial and simplified nature of these environments may not fully capture the complex interplay of factors that influence thermoregulation in the wild. For example, the absence of predation risk (Rusch, Sears & Angilletta, 2023), social interactions (Brien et al., 2012; Seebacher & Grigg, 1997), the influence of water availability/ hydration state (Sannolo & Carretero, 2019), or fluctuating environmental conditions (Taylor et al., 2020), all of which can lead to discrepancies between laboratory and field-observed parameters and behaviours. Such mismatches emphasize the

importance of integrating laboratory findings with field data to ensure ecological relevance. Field validation of laboratory results can reveal how thermal preferences align with habitat use and whether laboratory conditions accurately reflect the natural constraints that reptiles face.

In addition to measuring body temperatures, it is essential to quantify the thermal environment reptiles inhabit. Operative temperatures (T_e) describe the range of thermal conditions available to an organism in its habitat and serve as a benchmark for comparing environmental temperatures to the animal's physiological thresholds and behavioural preferences (Angilletta, 2009). They bridge the gap between environmental variability and reptile thermoregulation. By comparing T_e to T_{pref} or to T_{set} (or even the critical or voluntary thresholds), for instance, researchers can assess whether a habitat provides adequate or restricted thermal resources, or whether behavioural and/physiological thermoregulatory adjustments are necessary, or even possible (Diele-Viegas et al., 2018; Virens & Cree, 2019). For example, desert reptiles often face thermal landscapes where operative temperatures during the hottest times of the day will far exceed an animal's T_{pref} (Grimm-Seyfarth, Mihoub & Henle, 2017; Laura-Resendiz et al., 2014; Tatu, Dutta & Thaker, 2024), or sometimes even voluntary or critical thermal limits (Camacho et al., 2024). In such cases, behavioural strategies of thermal avoidance (Bawens, Hertz, & Castilla, 1996; Seebacher 1999), such as seeking shade (Kearney et al., 2012) or burrowing (Moore, Stow & Kearney et al., 2018), along with physiological adaptations (Seebacher & Franklin, 2005) that prevent overheating, become critical for avoiding heat stress. Conversely, in cooler habitats, reptiles may face limited opportunities to achieve their T_{pref}, relying heavily on basking opportunities or substrate selection (Carrascal et al., 1992; Dzialowski & O'Connor, 2001b) as well as physiology that maximises heat acquisition and retention (Bodensteiner et al., 2020), to compensate for environmental thermal deficits. Ultimately, in both extreme ends of the environmental spectrum, behavioural and physiological adaptations of reptiles enable these animals to still exploit some of these habitats (Narayanan, 2025; Cloudsley-Thompson, 1991).

Hence, mapping a habitat's thermal landscape may prove paramount to understanding daily activity patterns, hours of restriction and the inherent costs of thermoregulation for an animal in such habitat (Sears et al., 2016). For this, the integration of environmental sensors with operative temperature models is essential. Instruments such as thermocouples, pyranometers, and anemometers measure variables like ambient temperature, solar radiation, and wind speed, respectively, providing a multidimensional view of the thermal environment (Hall & Warner, 2019; Ortega, 2016; Spears et al., 2024). These data may be particularly valuable for assessing the impacts of habitat modification and climate change on reptilian thermoregulation. For instance, studies using operative temperature models have revealed

how deforestation and urbanization alter thermal landscapes, potentially to the extent where they exceed the adaptive capacity of thermally specialized species (Nowakowski et al., 2016; 2018; Rossigalli-Costa & Kohlsdorf, 2022; Vaughn et al., 2023). Conversely, though, other studies have shown how some species are able to survive (even if not always strive) in such modified landscapes (Frishkoff, Hadçey & Daily, 2015). They achieve this through either behavioural and/or physiological plasticity (Chevin, Lande & Mace, 2010), or sometimes even rapid evolution (Angilletta, Niewiarowski & Navas et al., 2002). Nevertheless, some thermal traits are more labile than others (Angilletta, 2009; Angilletta, Niewiarowski, & Navas, 2002), constraining the extent (Bodensteiner et al., 2020) and direction (increase vs decrease in temperature) (Sun et al., 2025) to which such adaptation can take place.

Ultimately, measuring body temperatures, determining thermal parameters and relating these to thermal landscapes, requires a careful balance between methodological rigour and ecological relevance. Traditional techniques, such as cloacal probes and physical operative models, remain indispensable for core temperature and thermal availability assessments, while modern tools like temperature-sensitive tags and anatomically accurate operative models provide dynamic and context-specific insights. Addressing challenges such as regional heterothermy and thermal inertia, while also incorporating environmental data ensures that these measurements capture the complexity of reptilian thermal ecophysiology. Together, these approaches provide a robust foundation for understanding how reptiles interact with their thermal environments and how they may respond to current and future environmental challenges.

1.3.2. From Basic Instruments to Advanced Sensors: Biologging, Biotelemetry, and Integrated Sensor Arrays

The field of thermal ecology has seen a remarkable evolution in the tools and methodologies used to measure body temperature and thermal environments. From the early use of simple thermometers to the development of sophisticated biologging systems and integrated sensor arrays, these advancements have revolutionized our understanding of thermoregulation (Taylor et al., 2020), with many such studies focusing on lizards and crocodilians. This progression reflects the growing recognition of thermal complexity in natural habitats and of reptile thermal physiology, as well as the need for tools that can capture dynamic, high-resolution data while minimizing disturbance to animal subjects (Barroso et al., 2016; Sannolo et al., 2014). This section explores this evolution, emphasizing how each technological step has expanded our ability to study reptiles, especially in their natural environments.

The first tools employed in reptile thermal ecology were mercury thermometers and thermocouples. Mercury thermometers, used extensively in the mid-20th century, provided accurate but static, or punctual, measurements of core body temperature (Brattstrom et al., 1965; Taylor et al., 2020). Typically, these measurements were taken by inserting probes into the cloaca of a restrained animal (Angilletta, 2009; e.g. Clusella-Trullas et al., 2007; Sears et al., 2016). However, the invasive nature of this method, coupled with its poor ability to capture rapid temperature changes, limited its ecological relevance. Alternatively, thin (and often flexible) thermocouple probes, which convert temperature changes into measurable electrical signals, marked an improvement by allowing for very precise and repeated measurements during experiments or over very short timeframes. The length and flexibility of said probes allowed animals to be less restrained, although the trailing flexible cloacal temperature probe could still present a potential hindrance to the animal's natural behaviours (McMaster & Downs, 2013). Hence, ultimately these tools were still constrained by some reliance on direct animal handling (or at least, uncomfortable set-ups), which could disrupt natural behaviours and potentially skew results (Langkilde & Shine, 2006). Seminal studies on lizards relied heavily on such tools to establish early correlations between environmental temperatures and body temperature regulation (Brattstrom, 1965). Furthermore, many T_{pref} and other thermoregulatory thresholds in reptiles have historically also been established through these methods (Angilletta, 2009; Camacho & Rusch, 2017).

A significant leap forward came with the advent of temperature-sensitive transponders, which allowed for [often continuous] monitoring of internal body temperatures in a minimally invasive manner (Goldman et al., 2004; Roark & Dorcas, 2000). These devices are often implanted subcutaneously, intramuscularly or, less often, intraperitoneally (Johnson, Webb, & Tanner, 1976; Langer & Fietz, 2014; Roark & Dorkas 2000). They are able to transmit temperature changes in real-time, offering insights into animals' diel and seasonal thermal fluctuations (Langer & Fietz, 2014). For example, subcutaneous transponders have been used in studies of torpor or hibernation/brumation in small mammals (Langer & Fietz, 2014) and reptiles, such as geckos (Nordberg & Schwarzkopf, 2019), revealing fine-scale temperature patterns during periods of dormancy (Clusella-Trullas & Chown, 2014). The use of such transponders across a range of taxa has successfully highlighted the thermal stability achieved through both aquatic and terrestrial thermoregulation, providing data crucial for understanding habitat preferences and energy budgets, even when animals are inside refuges during daily/seasonal periods of inactivity (Ariano-Sanchez et al., 2022; Perry, Gangloff & Rutschmann, 2025; Perry et al., 2025).

Building on the functionality of transponders, data loggers further enhanced the capacity to record temperature continuously and autonomously over extended periods (Virens & Cree, 2022). Unlike earlier tools, data loggers can store large datasets, capturing long-term thermal trends without requiring constant human intervention or the need to be within a certain distance range from a receiving station (Langer & Fietz, 2014) – although increasingly loggers have the dual ability to record and transmit the recorded data wirelessly. These devices have been transformative in studying elusive or highly mobile species, where repeated manual measurements or continuous data transmission are impractical or impossible. For example, several studies on multiple crocodilian species have used implanted loggers to monitor core body temperatures across diverse environmental conditions, revealing how these reptiles exploit thermal gradients in both aquatic and terrestrial habitats to optimize thermoregulation (Bassetti et al., 2020; Campos & Magnusson, 2012; Downs, Greaver, & Taylor, 2008; Grigg et al., 1998; Seebacher & Grigg, 1997; Smith, 1979), while button-sized loggers attached to the shells of turtles have provided insights into the interplay between behaviour, activity and thermoregulation, linking activity patterns to environmental constraints (Dall'Antonia et al., 2001).

The invention of telemetry systems marked another critical advancement, enabling researchers to pair thermal data with spatial and behavioural information. Radio telemetry, one of the earliest forms of this technology, relied on triangulating a tagged animal's position through a radio signal transmitted from the tag in the animal (Simbula et al., 2025b). Depending on the study, the signal itself could also encode further information such as a measure of [body] temperature (e.g. Hocutt, 2022). When in range of the receiver antennas, the signal could then be decoded to obtain such information and the animal located often by resorting to a "boots on the ground" approach to triangulate the source location of said signal (Crane et al., 2021). However that is usually a labour intensive endeavour (Crane et al., 2021) that may prove complex depending on the environment in question - due to accessibility (Taylor, DeNardo & Malawy), signal attenuation (Taylor et al., 2020), size of the animal's ranging patterns (Harlow et al., 2010), etc. Nevertheless this tool has been widely and successfully used to track the movement and, in some cases, also the thermal profiles (Lutterschmidt & Reinert, 2012), of many animals including reptiles (often large species such as monitor lizards) (Harlow et al., 2010; Simbula et al., 2025b), crocodilians (Mascarenhas-Junior, Sousa Correia & Simões, 2023) and even some snakes (Shine & Lambeck, 1985) and chelonians (Price et al., 2022). More recent advances in telemetry systems have allowed monitoring long-ranging aquatic species like estuarine crocodiles (*Crocodylus porosus*), providing continuous data habitat use (Brien et al., 2008).

GPS-integrated telemetry systems have simplified the triangulation process (i.e. done automatically), increased the range of action and added a degree of spatial and temporal resolution beyond what would be feasible with traditional radio telemetry (Smith et al., 2018). This has helped further reveal how reptiles navigate their landscapes and even select macro- and/or microhabitats (Price-Rees, Brown & Shine, 2013). Furthermore, the integration of multiple sensors, such as magnetometers, gyroscopes and tri-axial accelerometers, have further enhanced the utility of telemetry by capturing behavioural in addition to spatial data (Zotos et al., 2025). Nevertheless, few studies [in reptiles] pair this ability to analyse spatial data alongside thermal measurements of body and/or environmental temperatures. One rare example of a study comparing these combined accelerometry with temperature loggers to quantify the energetic costs of thermoregulation on monitor lizards, shedding light on the trade-offs between activity budgets and thermal stability in resource-limited environments (Wild et al., 2025). While similar studies resorting to these integrated solutions already exist for other taxa (e.g. sharks - Waller et al., 2023) the field of reptile thermal ecology is yet to fully explore the use of these novel tools.

Still, all the aforementioned tools require some level of interaction of the animal, even if just to implant/deploy the logger/sensor/transmitter/tag. Contrastingly infrared thermography (IRT) and thermometry now presents another increasingly accessible set of tools for studying fine thermoregulation patterns, offering non-invasive, real-time measurement of surface temperatures [usually] without the need to touch the subject (Barroso et al., 2016; Carretero, 2012; Chukwuka, Virens, & Cree, 2019). IRT cameras detect infrared radiation emitted (and reflected) by a body, producing detailed thermal maps that can reveal regional heterothermy (Tattersall, 2016) and heat exchange dynamics (Sannolo et al., 2014), even in very fine temporal resolution – through radiometric video, for example. These capabilities have been particularly valuable in studying small or cryptic species, such as lacertid lizards (Barroso et al., 2016; Luna, Pérez i de Lanuza, & Font, 2013), where traditional methods are often impractical. For example, IRT has been used to study the thermal impact of digestion in iguanas (Rusu et al., 2016). Thermography has also facilitated studies on crocodilians, revealing how fine and fast vascular adjustments regulate surface temperatures during basking and submersion (Clarac et al., 2017; Clarac & Quilhac, 2018).

These technologies have not only refined the study of reptilian thermal ecology but have also informed broader applications. For example, data collected using thermography and biologging devices have been used to improve husbandry practices in laboratory and captive settings (Goodwin, 1998; Quimby, Olea-Popelka & Lappin, 2009). Insights into diel and seasonal thermal preferences have informed the design of enclosures with gradient-based

heating systems, ensuring that captive reptiles have access to conditions that mimic their natural habitats (Giacometti et al., 2023; Denommé, Bakker, & Tattersall, 2025). Similarly, studies using IRT and accelerometry have enhanced the ecological relevance of experimental designs, allowing researchers to replicate the thermal and behavioural dynamics observed in the wild (Barroso et al., 2016).

1.3.3. Infrared Thermometry and Thermography – Principles and Applications

Infrared-based tools offer non-invasive and precise methods for measuring surface temperatures. The principles of infrared radiation, which is emitted (and, to some degree, reflected) by all objects with temperatures above absolute zero (Harrap et al., 2018; Speakman & Ward, 1998; Tattersall, 2016), form the foundation of both infrared thermometry and thermography. These technologies rely on sensors to detect the intensity of emitted (and reflected) infrared radiation and convert it into temperature estimates. While both are grounded in similar principles, their applications, strengths, and limitations differ substantially. Infrared thermometry involves single-point measurements (Carretero, 2012; Chukwuka, Virens & Cree, 2019), where a sensor detects radiation from a defined area on an object's surface. This method is rapid, portable, and ideal for quick assessments, making it useful for applications requiring immediate temperature readings (Tattersall, 2016). However, its utility is limited by its lack of spatial and temporal resolution, providing no insight into temperature distributions across an object's surface (Barroso et al, 2016; Taylor et al., 2020). Furthermore, typical IR thermometer "guns" are often difficult to use accurately since the exact location from where the reading is being measured is difficult to precisely locate, as there is no direct way to visualize the measured area (Carretero, 2012; Playà-

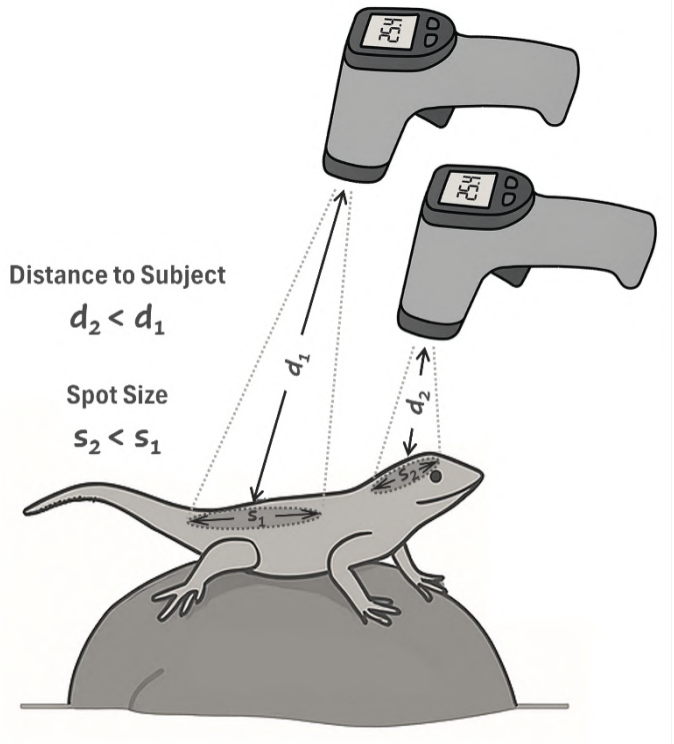

Fig. 1.5: *Diagram exemplifying the relationship between spot size and distance between the subject and the infrared thermometer (or thermal camera). Image credit: Frederico M. Barroso; AI-assisted elements*

Montmany & Tattersall, 2021) - besides often a single point given by a laser-pointer (Fig. 1.5). Nevertheless, the spot given by such laser-pointer is not necessarily representative of the actual area being measured and averaged into a single temperature reading (Carretero, 2012; Chukwuka, Virens & Cree, 2019). Hence, these tools are highly susceptible to biases induced by spot size effects (Tattersall & Playà-Montmany, 2021) (Fig. 1.5). Furthermore, although many such tools do enable accounting for emissivity of the material, they often do not allow accounting for any other parameters which are crucial for accurate temperature estimates (Tattersall, 2016, Taylor et al, 2020). In fact, studies are divided regarding the usefulness and accuracy of these tools in inferring body temperature of small lizards (Carretero, 2012; Chukwuka, Virens & Cree, 2019). Nevertheless, it is mostly ubiquitously accepted that, in order to obtain the most accurate measurements of body temperature using these tools, measurements should be taken as close to the subject as possible (Carretero, 2012). However, this leads to the need for potentially disruptive, stress-inducing interactions (including direct handling of the animal such as that in the study by Chukwuka, Virens & Cree, 2019), thus defeating one of the main advantages of IR-based measurements – the reduced invasiveness of non-contact temperature measurements (Speakman & Ward, 1998).

Infrared thermography (IRT), in contrast, maps temperatures across all surfaces within the camera's field of view (FOV), generating thermal images, or "heat maps" (Goller, Goller & French, 2014; Speakman & Ward, 1998; Tattersall, 2016). Cameras equipped with microbolometer arrays detect infrared radiation, assigning temperature values to each pixel to produce detailed spatial representations of thermal gradients (Tattersall, 2016). This capability allows researchers to assess regional heterothermy (Barroso et al, 2016; Sannolo et al., 2014), monitor dynamic temperature changes, and identify small-scale thermal patterns in their studied subjects and their surrounding surfaces simultaneously (Blais et al., 2023; Goller, Goller & French, 2014). For example, a thermal image of a lizard basking can reveal variations between the head, body, and limbs, as well as providing a context of the thermal landscape around it (e.g. the temperature of the substrate it sits on) (e.g. Luna, Pérez i de Lanuza, & Font 2013). Ultimately this provides insights into thermoregulatory strategies that cannot be captured by single-point, contact or non-contact, thermometry. Additionally, radiometric (i.e. thermal) images can be processed further using a range of available software which provide an increased flexibility of adjusting for important parameters well beyond just the surface's emissivity (Barroso et al., 2016). Such corrections can be applied on a pixel-by-pixel basis, allowing precise adjustments to be made for different measurements taken from different surfaces on the same radiometric photo (Faye, Dangles & Pincebourde, 2016).

Still, accurate infrared measurements depend on properly understanding factors (Fig. 1.6) like emissivity, ambient reflective temperature, environmental attenuation, and the spot size effect, many of which are still often disregarded even in current thermal ecology studies resorting to this tool, but which must be accounted for when inferring real temperature from a thermal photo (Harap et al., 2018).

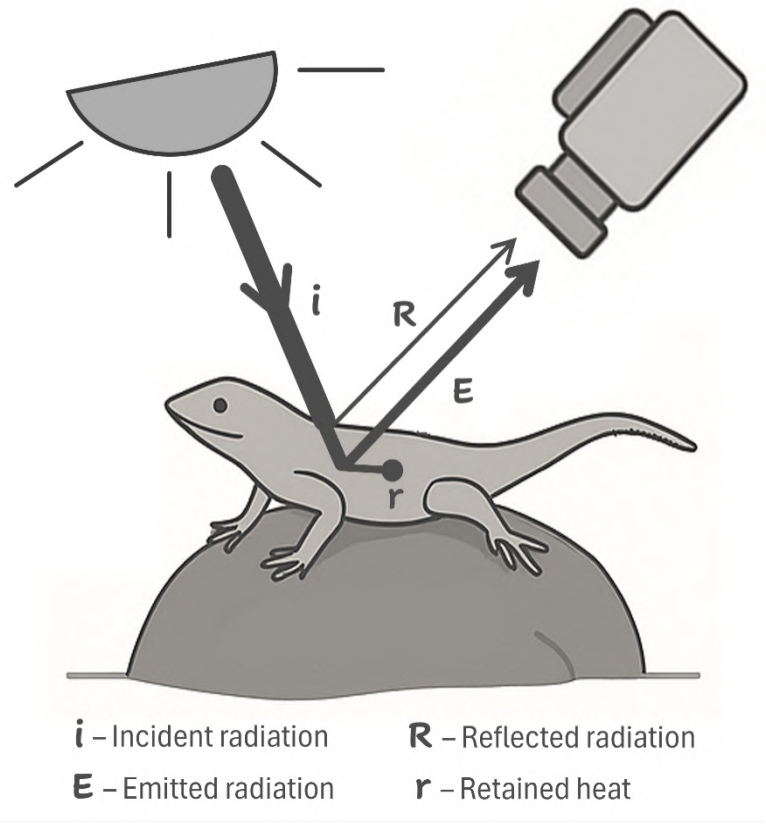

Fig. 1.6: *Diagrammatic representation of the basic principles behind estimation of temperature with correction for emissivity and ambient reflective temperature. Where the thermal camera receives a combination of reflected and emitted radiation from the subject but also not all the thermal energy in the subject will be emitted back from the body, depending on its emissivity. Image credit: Frederico M. Barroso; AI-assisted elements (ChatGPT, OpenAI)*

The most immediate variable that must be accounted for is emissivity (Ɛ). This is the proportion (as a ratio on a scale of 0 to 1) of thermal energy emitted from a material's surface compared to that emitted from a black body under similar conditions (Taylor et al., 2020). Biological surfaces, including reptile skin, typically have high emissivity values (around 0.96 - Tattersall, 2016), meaning they will re-emit most of the infrared radiation they absorb/ contain. However, surface characteristics, such as roughness or moisture (Playá-Montmany & Tattersall, 2021), and the angle of incidence can alter apparent emissivity, leading to inaccurate temperature estimates if not properly considered or calibrated for. For instance, studies on lizards and snakes have shown how emissivity declines at steep angles, significantly underestimating temperatures when viewed from oblique perspectives (Playá-Montmany & Tattersall, 2021). Nevertheless, emissivity is the parameter that is less often disregarded when resorting to thermography for measuring body temperature of animals. Still, the angle of the photo should be more often considered.

Another important variable is the effect of ambient reflective temperature. Correcting for this is necessary since infrared cameras can pick up reflected infrared radiation from nearby

objects or the environment, which can skew – often inflating - temperature readings of the desired surface (Taylor et al., 2020). Correcting for reflected temperatures is crucial for accurate and consistent data collection. This is particularly important in outdoor contexts where the surrounding environment, and hence the radiant heat sources and their intensities, are more dynamic and thus any error introduced into the data by not correcting for these sources would likely not be a systematic one, affecting data comparability even within the study (Tattersall, 2016).

Furthermore, there is an inherent level of environmental attenuation that must be accounted for or, at least, minimised/ standardised as best as possible. This attenuation happens due to factors like humidity and atmospheric particles which absorb and scatter infrared radiation (Fig. 1.7), leading to reduced measurement accuracy with increasing distance between the camera and the subject (Faye, Dangles, & Pincebourde, 2015; Taylor et al., 2020). Cameras or IR photo processing software rely on algorithms to compensate for these effects, but they often require some level of user input (such as inputting a measured distance between the camera and the subject, or the relative humidity at the time of capturing the photo) that should not be disregarded (Faye, Dangles & Pincebourde, 2016). Methodological considerations can also aid in reducing the impact of these by attempting to maintain environmental conditions as stable as possible as well as minimising and, above all, maintain the distance between the camera and the subject constant (Tattersall, 2016). However, while this may be relatively simple in a laboratory context, it is seldom the reality in the field. Finally, extreme conditions, sometimes observed in variable field contexts, can introduce significant variance or errors in the measurements obtained.

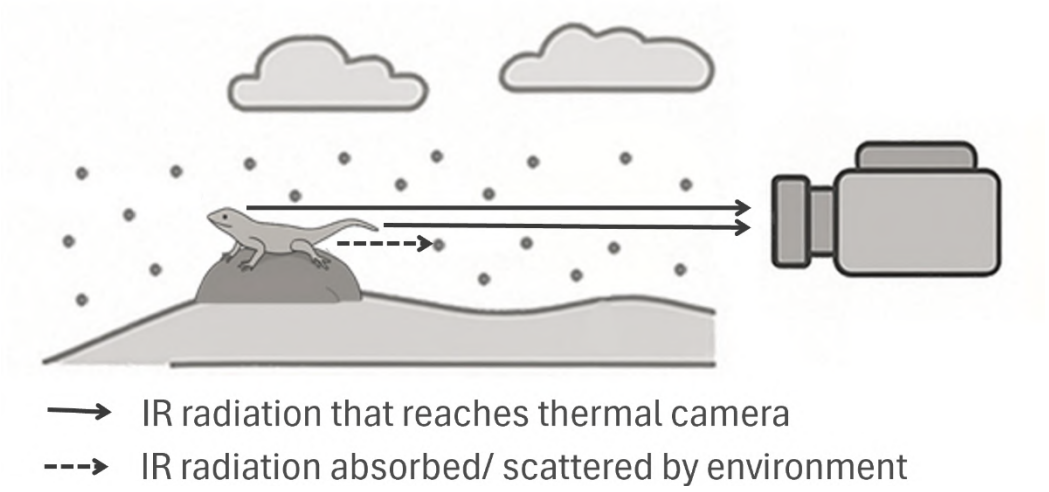

***Fig. 1.7**: Diagrammatic representation of the effect of environmental attenuation by particles, such as air and humidity. IR radiation emitted from the animal will either reach the camera sensor or get absorbed or scattered by environmental particles. The larger the distance between the subject and the camera, the higher the environmental attenuation due to increased opportunity for scattering or absorption.* Image credit: Frederico M. Barroso; AI-assisted elements (ChatGPT, OpenAI)

Lastly, another variable that is an inherent consequence of the distance between the camera and the subject, as well as the resolution of the camera itself, is the “Spot Size Effect”, already discussed prior in the context of pyrometers (IR thermometry). The spot size is the smallest area a camera can accurately measure at a given distance (Playà-Montmany & Tattersall, 2021). In other words, it is the realised area (on the subject) that each pixel represents. The larger the pixel or, more relevantly, the bigger the distance between the subject and the camera, the larger the effective area on the subject will be represented in each pixel. Hence, for detailed measurements, such as capturing the surface temperature of a reptile’s eye or limb, maintaining an appropriate distance-to-spot size ratio is critical to ensure that the measured pixel is representative of the target area and not an average of the area in question and surrounding surfaces (Chukwuka, Virens & Cree, 2019). If the target is smaller than the spot size, the temperature reading will pertain to a non-representative average of the entire spot, blending the target with the surrounding background. Again, consistency is key to ensure accuracy, adequate precision and comparability of the measurements.

However, infrared thermometry and thermography have broad applications that span beyond the field of thermal ecology research, with ample uses in areas ranging from veterinary diagnostics to agricultural monitoring and wildlife monitoring and research (e.g. Blenkus et al., 2022; Medina et al., 2019; Rzucidlo, Curry, & Shero, 2023; Tattersall, 2016; Vollmer & Möllmann, 2018). In veterinary medicine, thermography is used to detect inflammation or injury by identifying localized "hot spots" on an animal's body (Shecaira et al., 2019). In agriculture, thermal imaging helps monitor plant health by assessing stomatal conductance and evapotranspiration rates (Mertens et al., 2023). These tools have also been employed in conservation biology to locate and monitor cryptic species, such as bats, nocturnal birds or amphibians, based on their heat signatures (Dawlings et al., 2023; Wagner et al., 2025). In wildlife biology, infrared tools are used to study thermoregulation in diverse taxa. For instance, thermography has been applied to monitor temperature variations in bird feathers during flight, revealing how avian species manage heat dissipation (Crandell, Powers & Tobalske, 2025). Similarly, IR tools have been employed to estimate core body temperatures in mammals by targeting thermally active regions, such as the periorbital area or nasal passages (Casas-Alvarado et al., 2022).

In thermal ecology specifically, these tools have been transformative, particularly in studying reptiles. IRT, with its ability to capture whole-body temperature distributions, is invaluable for understanding regional heterothermy (Barroso et al., 2016). Studies on monitor lizards (*Varanus spp.*) have used IRT to examine how specific body parts heat and cool at different rates, providing insights into behavioural and physiological mechanisms of thermoregulation

(Mahfud et al., 2025). Similar studies on crocodilians have demonstrated the role of vascular shunting in modulating heat transfer, showing how fast blood flow adjustments enable these reptiles to maintain core temperatures while basking or submerged (Clarac et al., 2017; Grigg & Kirshner, 2015).

The integration of thermographic data with laboratory-based measurements has bridged gaps between controlled and field conditions (Taylor et al., 2020). In thermal gradients, reptiles exhibit behavioural preferences that correspond to specific temperature ranges. Mapping these preferences using IRT in natural habitats validates these laboratory findings, ensuring ecological relevance (Blais et al., 2023). For example, combining thermography with biophysical models has enabled researchers to estimate operative temperatures with unprecedented accuracy, incorporating real-world variables like wind speed and solar radiation (Bakken & Angilletta, 2013).

Improved calibration and validation protocols have enhanced the reliability of infrared tools in field applications (Barroso et al., 2016). Using blackbody [and other] references, researchers can standardize camera settings, or radiometric image processing pipelines, to account for emissivity and environmental effects, minimizing error (Tattersall, 2016). Similarly, adopting radiometric, lossless data formats preserves the raw infrared information, allowing for post-capture analysis and reducing biases in temperature estimation (Franco et al., 2019; Taylor, 2020).

Infrared thermometry and thermography represent powerful tools for understanding thermal processes across biological systems. Their non-invasive nature, combined with their ability to capture fine-scale thermal patterns, has made them indispensable in wildlife biology, veterinary sciences, and, especially, thermal ecology. By addressing factors such as emissivity, spot size, and environmental attenuation, studies can maximize the accuracy and applicability of these tools, providing critical insights into the complex thermal landscapes that organisms navigate. These advancements continue to inform the study of reptile thermal biology, offering a foundation for the integrative approaches explored in this thesis.

1.4. Thesis Framework and Objectives

The evolution of the tools and methods used in thermal ecology studies underscores the importance of continuous innovation. Each technological advancement has addressed limitations of earlier tools, offering deeper insights into the complexities of thermoregulation, thermal physiology and habitat use. Still, the peril of the increasingly diverse toolkit available to thermal ecologists, lies on the easily indiscriminate use of such tools. Especially without carefully acknowledging the historical research done in the field, or considering the implications of deviating from the standard measurements – even if more rudimentary - obtained via tools and methods ubiquitously deployed throughout the field's history.

For instance, this diversification of tools and methods, leads to an important, somewhat philosophical, question that must be considered at the embryonic stage of any novel thermal ecology study: what is THE body temperature of an animal? Indeed, the pioneering works by Heath in the 1960's already hinted at the fact that there may not be such a thing (i.e. a single body temperature, representative of core state, does not exist), as even small lizards can exhibit marked patterns of regional heterothermy. Still, cloacal temperature has, historically, been ubiquitously set as the standard location for inferring core state (Taylor et al., 2020). This choice is perhaps an artefact of the tools available at the time when this field of studies emerged. Nevertheless, since then, the literature has been populated with plentiful important and field-defining works relying on this measure of body temperature as a standard. Hence, for decades, thermal ecologists have been defining and comparing thermal physiological parameters of species based on the standardly measured cloacal temperature, ensuring inter-study comparability of results, and contributing towards a unified field.

All this considered, it is important to carefully take into account the way of integrating innovative tools and methods into this field of studies. More research is required to test the cross-comparability of the temperatures obtained via different tools and/or across different body regions and establish calibration curves, whenever historical comparability is desired. Hence, while new tools can provide powerful insights into new and old questions regarding reptile thermal ecology, one cannot disregard the fact that many standard thresholds and parameters have been established otherwise. To address this, either new standards need to be agreed upon - in itself a Herculean task - or calibration curves, which enable cross-study comparability, need to be established. Either way,

decades of important thermal ecology research should not be disregarded when faced with newer, more flexible/ precise tools such as thermal imaging.

Nevertheless, by capturing dynamic and multidimensional data, modern tools have bridged the gap between laboratory studies and field observations, paving the way for integrative approaches that combine behaviour, physiology, and environmental variability. These advancements provide a robust foundation for the chapters of this thesis, which leverage these tools to address longstanding questions about reptilian responses to changing thermal landscapes. Specifically, this thesis addresses the important question on how to capitalise on such technological advancements – testing several options - to obtain precise, minimally invasive and comparable (to historical standards) measures of body temperatures in reptiles. It dwells on the issue of finding new standards locations to infer internal state, and how ubiquitous such standards are across different body sizes and even reptile groups – with work done on two contrasting animal groups, small lizards and large crocodiles. Furthermore, the chapters of this thesis also dive deeper into some of the new tools available to the modern thermal ecologist, further discussing the advantages and disadvantages of said tools. Additionally, this work also aims to provide actionable suggestions to improve captive/laboratory husbandry of test subjects, acknowledging the fact that stress (regardless of induced through the use of invasive measurement tools/practices, through husbandry practices, or through experimental set-ups) will have an inherent effect on an animal's behaviour, and hence, on a reptile's [behavioural] thermoregulation.

The following [second] chapter of this thesis builds on previous work by Barroso et al (2016) demonstrating the applicability of infrared thermography to infer internal body temperature of lizards. Unlike former studies which focused on diurnal, heliothermic lacertid lizards, this chapter studied the Moorish gecko, *Tarentola mauritanica*, which exhibits very different habits, thermal strategy and physiology, to that of previously studied species, thus exploring this tool's ubiquitous potential. Specifically, the chapter further emphasizes the applicability of the eye as a new, yet retrospectively comparable (given adequate calibrations), standard location to infer core temperature, yet through non-contact tools such as thermal imaging.

The applicability of thermal imaging is then further explored in the third chapter. This time, radiometric video, allied to the concept of regional heterothermy, are utilised to investigate thermal exchange rates (once more utilising *Tarentola mauritanica* as test species) at a very fine temporal and spatial (i.e. of different body parts) scales. This chapter suggests a novel and flexible set-up that allows the thermal exchange rates of

small lizards to be measured in a laboratory context, with minimal invasiveness to the lizard. The flexibility of the set-up allows to explore the effect of different heating regimes (such as heliothermy and thigmothermy, but not exclusively) in the thermal exchange rates and heat distribution (and hence, pattern of regional heterothermy) along the body of a semi-freely thermoregulating lizard. The modular and relatively simple set-up, devised and tested in this chapter, represents a suitable and less invasive alternative method to establish thermal exchange rates of lizards under controlled laboratory conditions.

The fourth chapter, on the other hand, revolves around the similar underlying question as chapter 2, however in a much larger animal (the Morelet's crocodile, *Crocodylus moreletii*) with an amphibious lifestyle. In this chapter several tools and methods are field tested to explore patterns of regional heterothermy in crocodilians while defining suitable alternative proxies of internal body temperature in animals with large body size range. The study involved testing a combination of different types of thermal sensors, such as thermal imaging and a range of data loggers, deployed internally, subcutaneously or superficially across different body parts of the crocodiles. All these with the aim of establishing how well the pattern observed in Chapter 2 (and other studies by Barroso et al, 2016) scales up to an animal of a large body size, and if indeed similarly body locations as the eye still provide informative proxies of core temperatures. Additionally, this chapter is meant to represent a starting step towards devising an integrated tagging solution that will enable the integration of body temperature/ thermal ecology data along with spatial (GPS), behavioural (accelerometery, magnetometry) and environmental data, currently in the R&D stages, as a direct consequence of the work presented here.

Finally, the fifth chapter focuses on the effect of captivity on the behaviour of test subjects. The work arose from an evident gap in the literature of adequate protocols for brumating lizards in captivity and the awareness that inadequate husbandry conditions would inevitably affect an animal's behaviour, and hence, potentially skew results of subsequent thermal ecology studies, on animals whose thermoregulation is primarily behavioural. Hence, this chapter presents a detailed protocol devised for safely brumating two lacertid lizard species in laboratory settings, and will hopefully contribute towards better and more standardized husbandry practices of study subjects.

Ultimately, the chapters of this thesis aim to rigorously examine and test new and emerging tools in the field of reptile thermal ecology, proposing some stepping stones towards novel standardised methodologies that capitalise on the advantages of these

emergent technologies, whilst acknowledging the need for cross-study comparability, historical or otherwise, as further synthesised in the sixth chapter.

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2. Chapter 2 - Evidence from *Tarentola mauritanica* (Gekkota: Phyllodactylidae) helps validate thermography as a tool to infer internal body temperatures of lizards

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2.1. Abstract

Infrared (IR) thermal imaging has become an increasingly popular tool to measure body temperature of animals. The high-resolution data it provides with short lag and minimum disturbance makes it an appealing tool when studying reptile thermal ecology. However, due to the common phenomenon of regional heterothermy and surface-to-core temperature gradients, it is essential to select the appropriate body part to measure and provide calibrations to accurately infer internal body temperatures. This work follows from a previous study on lacertid lizards to assess the reliability of thermography-measured body temperatures, from several body locations, as a proxy for internal body temperature in lizards. This study focuses on the Moorish gecko, *Tarentola mauritanica,* due to its distant phylogenetic relationship and its different ecology and morphology from the previously tested species. A total of 60 adult geckos of both sexes and of a range of sizes were tested in thermal gradients and subjected to a sequence of randomly assorted treatments of heating and cooling. The temperatures of the animals were periodically measured with a thermal camera at six different body parts and, immediately after, the cloacal temperature was then measured with a thermocouple probe. Body parts' temperatures, obtained thermographically, were regressed against cloacal temperature using OLS regression and the pairwise correlations were tested using Spearman coefficients. Relationships among all body parts and between all body parts and the cloaca were strong in all cases ($R^2 > 0.87$, Spearman Correlation > 0.95). The observed pattern was very similar to those previously obtained from lacertid lizards. Ultimately, the eye proved to provide the best overall proxy for internal temperature, when accounting for both the slope and intercept of the regression. Hence, this study provides further support for the establishment of the eye as the standard location to infer internal body temperatures of lizards through thermography.

2.2. Introduction

The field of thermal ecology has, through the years, provided abundant important insights into the ecology, physiology and diversity of reptiles (Angilletta et al., 2002; Huey and Stevenson, 1979; Savage et al., 2004). The literature is laden with studies demonstrating the fundamental role of temperature for the ecophysiology of reptiles (e.g. Seebacher and Franklin, 2005) and on how body temperatures are maintained through behavioural adjustments, physiology and microhabitat selection (Angilletta et al., 2010; Brown et al., 2004; Goller et al., 2014). This ability allows reptiles to optimise a range of metabolic

functions (Angilletta et al., 2004; Angilletta et al., 2010; Huey and Stevenson, 1979) such as locomotion, digestion, growth and reproduction, without the high costs of endogenous heat production endured by endotherms (Huey and Slatkin, 1976).

The pivotal role that temperature plays in the ecology of reptiles led to the early origin of now well-established protocols for measuring internal body temperatures. Cloacal probes, in use since the onset of this area of research (Angilletta et al., 2010), are a reliable tool that provide easily reproducible data with great accuracy and precision (e.g. Hibok 18 thermometer and thermocouple probe, used in this study, has an accuracy of ±0.2% and precision of 0.1ºC). However, as technology progresses, there has been an increasing reliance on new tools and protocols such as temperature-sensitive RFID (radio) tags (Roark and Dorcas, 2000), implantable data loggers (Campos and Magnusson, 2013), infrared thermometers (pyrometers) (Carretero, 2012; Chukwuka et al., 2019; Hare et al., 2007) and infrared thermal imaging (Bosch, 1983; Burns et al., 2015; Jones and Avery, 1989) to measure body temperatures. These allow high-resolution temperature data to be recorded with great speed and short lag while also minimising the animal disturbance (Barroso et al., 2016; Sannolo et al., 2014; Tattersall and Cadena, 2010).

Several studies have described the potential uses and advantages of said tools (Berg et al., 2015, Tattersall and Cadena, 2010; among others). Such is the case for infrared thermal imaging (hereafter referred to as thermography) where authors like Kastberger and Stachl (2003), Luna et al. (2013), Tattersall and Cadena (2010), and Virens and Cree (2019) demonstrated the potential uses of thermography for a range of studies in several taxa. However, few are the studies which evaluate the reliability of the data collected by this technology or its comparability to temperature data collected by other tools. An example would be the study by Carretero (2012) who demonstrated that temperatures of small lacertid lizards measured at a distance of 20 cm using a pyrometer (infrared thermometer) showed a non-isometric bias and a weak correlation to those obtained by cloacal probe, thus showing that pyrometry may not be a very reliable method of inferring internal body temperature. A more recent study by Chukwuka et al. (2019) with geckos obtained contrary conclusions to those reported by Carretero (2012) regarding the reliability of pyrometers but, in this case, measurements were taken at a distance of 5 mm, which required the animals to be handled. Indeed, these new tools are now widely used to produce increasingly complex results (e.g. Tattersall et al., 2016). As such, there is an urgent need to validate the use of these new instruments, as well as to

develop up-to-date, standardized methodologies in order to provide reliable as well as comparable results.

Two previous studies, by Barroso et al. (2016) and Sannolo et al. (2014), have shown that thermography can be reliably used to infer internal body temperature of small to large lacertid lizards. In Barroso et al. (2016), the authors capitalised on the phenomenon of regional heterothermy - a widespread phenomenon among lizards, initially described by Heath (1964) - to investigate which body part would consistently provide the best direct proxy for the internal body temperature (taken as the cloacal temperature). Barroso et al. (2016) concluded that the eye provides such opportunity and suggested possible explanations. These include the presence of large venous sinuses behind the eye, a common feature among many squamate groups including lacertids (Bruner, 1907), iguanids (Porter and Witmer, 2015) and even snakes (Bruner, 1907). As a result, lizards may in fact show particular care and precision in regulating head/brain temperature (King and Green, 1999; Porter and Witmer, 2015, Tattersall et al., 2006). Hence, if such a pattern was maintained across a range of phylogenetically distant reptile groups, the eye could prove to be a strong candidate to be set as a new standard location to measure internal body temperature of squamates through thermography.

Therefore, this study aims to further validate the use of thermography to infer internal body temperature and provide appropriate guidelines, now using the Moorish gecko *Tarentola mauritanica* (Gekkota, Phyllodactylidae) as a model. This is a small-sized species (maximum recorded snout-vent length of 86mm in the Iberian Peninsula), resulting in small thermal inertia (Bell, 1980) while, being a gecko, it is also phylogenetically distant from lacertids, iguanids and snakes (Pyron et al., 2013). As with most geckos, it also lacks eyelids, having a scale over the eye (the brille) instead (Bellairs, 2009) and it shows less marked occurrence of regional heterothermy (see Results). All these characteristics, along with the combination of diurnal and crepuscular activities and thus, alternating between heliothermic and thigmothermic strategies (Arad et al., 1997; Gil et al., 1994; Rato & Carretero, 2015; Simbula et al., 2019), makes it as distant as possible to a lacertid and hence, a good model to further test whether the pattern observed by Barroso et al. (2016) could potentially be ubiquitous among lizards.

Ultimately, the main goal of this study is to provide yet another stepping-stone towards the development of a unified protocol for the use of thermography as a non-invasive tool to measure body temperatures in reptile thermal ecology studies.

2.3. Methods:

2.3.1. Sampling and animal maintenance:

A total of 60 adults of *T. mauritanica* were tested in this study with a ratio of 31 males to 29 females, representing distinct body lengths (mean male SVL ± SD = 67.64 ± 7.99 mm; mean female SVL ± SD = 63.69 ± 4.62 mm) and body masses (mean male mass ± SD = 7.53 ± 2.18 g; mean female mass ± SD = 5.78 ± 0.92 g). *Tarentola mauritanica* was chosen due to its crepuscular and diurnal pattern of activity as well as its bimodal thermoregulatory strategy (i.e. heliothermy and thigmothermy, both observed in this species) (Rato & Carretero, 2015).

Animals were captured from four different populations (Torres Vedras: 39.09ºN, 9.25ºW; Évora: 38.56ºN, 7.91ºW; Portimão: 37.13ºN, 8.54ºW; Ayamonte: 37.21ºN, 7.38ºW) all belonging to the same mitochondrial lineage in order to avoid phylogenetic effects (Rato et al., 2012, 2016), but encompassing a wide array of environmental conditions from the Iberian Peninsula (Rato and Carretero, 2015). Geckos were collected in spring 2019 and kept in captivity in couples with water and food provided *ad libitum* throughout the period of captivity. The experiments were performed several months before the onset of the reproductive season to ensure all females were not in oogenesis or gravid.

2.3.2. Experimental settings:

Between the 25th of November and the 13th of December 2019, six experimental sets were carried out. For each one, a total of seven thermal gradients (± 16-55 ºC) set up in acrylic tanks (100 x 30 x 40 cm) with one 150W heat lamp placed at one end about 25 cm from the bottom and with an elevated aluminium plate running the full length of the terrarium as a refuge were set up following Rato & Carretero (2015). The heat lamp provided the opportunity for the animals to heat up heliothermically while also heating up the aluminium plate which in turn provided the opportunity for the animals to heat up thigmothermically. Therefore, with this set up, animals were able to vary between the two heating strategies as they have been reported to do in natural conditions (Arad et al., 1997).

Water was sprayed in the cold area before each experimental set started. This had the dual purpose of helping cool down the cooler side of the gradient, while also providing access to water as the level of hydration has been shown to affect thermoregulatory behaviour in some lizards (Sannolo & Carretero, 2019).

Males and females were previously labelled with dental floss loosely tied around the waist, and one couple was released per thermal gradient. The floss facilitated capture of the animal while minimising direct contact with it, which could interfere with the individual's temperature. Additionally, markings on the floss allowed the male and female individuals to be easily identified without the need to manipulate the animal. Order of individuals in the whole experimental setup was randomized, so individuals from different populations were tested in the same experimental set. Once all animals were in the thermal gradients, and in order not to just have warm or cold measurements, but to also ensure intermediate body temperatures were obtained, we used the android app *True Random Generator®* (downloaded from Google Appstore, 2019) to randomize the heat lamp state (on or off). In preliminary tests, it was observed that *T. mauritanica* heated up or cooled down quickly (from 18 ºC to 30 ºC in less than 3 minutes, from 30 ºC to 23 ºC in less than 4 minutes; authors, pers. obs.). Hence, 1 minute before the measurement each heat lamp was switched on or off according to the randomization. Heat lamp state was maintained until the following randomization event. This was done in order to obtain a uniform distribution of body temperatures over the widest possible temperature range. Measurements were carried out every 20 minutes until six recordings per individual were achieved. The experiments were performed in a large, air-conditioned room with air temperature set to 18ºC.

For the thermal pictures, 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 (assuming a skin emissivity = 0.96). IR camera was handheld and photos were shot at 30–40 cm distance from the animals. This approach allowed maintaining the same resolution in every IR image, irrespective of 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 a few millimetres into the cloaca of the animal.

In order to reduce possible measurement biases, FMB took the thermal pictures and GMR the cloacal temperatures, using the floss to catch the individual, avoiding direct contact as much as possible to prevent heat exchange between the animal and the measurer. At the end of each set of gradients, all subjects were weighed in a precision balance (Sartorius M-Pact AX224, Sartorius AG, Goettingen, Germany). After all the experiments, snout-vent length (SVL) was measured for each individual.

All IR images were processed using the software FLIR Tools 2.1 (Copyright 2014 FLIR Systems, Inc; http:// www.flir.com). The Spotmeter tool was used to extract the temperature dorsally at the centre of six body locations (Fig. 2.1): snout, eye, head, dorsum (centrally), leg (right hind knee articulation) and base of the tail (above the cloaca), as in Barroso et al. (2016). Whenever a body location was isothermic with the background (i.e. indistinguishable from the surroundings in the IR photo), the corresponding regular photo was used to try to locate the body part. The emissivity was set to 0.96 and the distance of measurement to 30 cm, while the ambient temperature and humidity were measured by the camera at the time of recording. For consistency, FMB processed all IR photos.

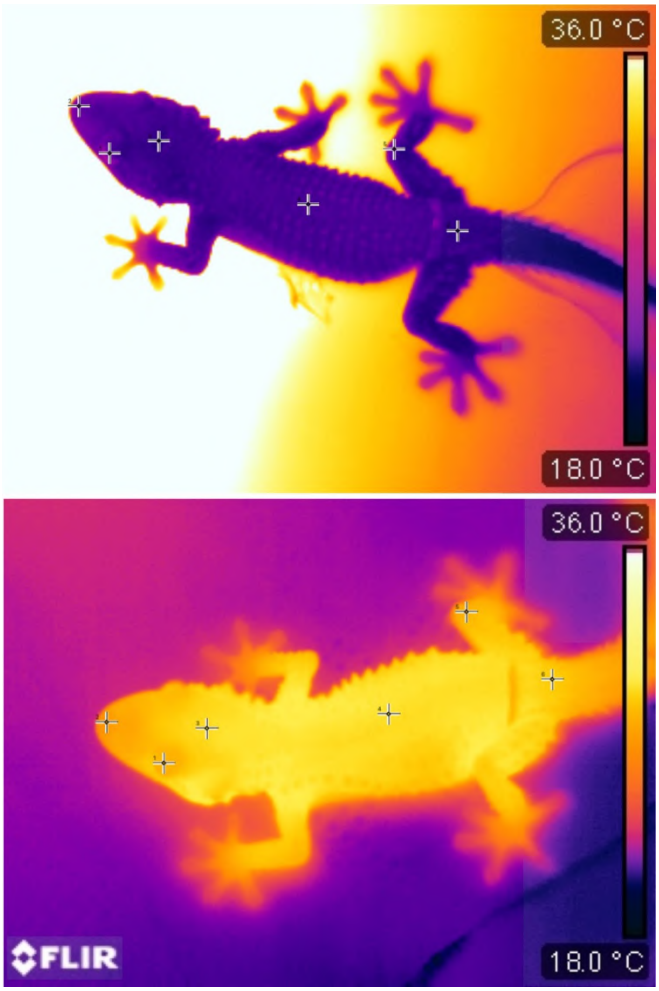

Fig. 2.1 - Infrared thermal photos of *T. mauritanica* showing the body parts measured and the low regional heterothermy observed both during heating (top) and cooling (bottom).

2.3.3. Statistical analysis:

We investigated the potential effect of trial number (six repetitions) and heating status (on or off) using a two-way interaction mixed-effects model (Mod1), with the gecko's individual identity set as random factor to account for the repeated measures and trial and heating status as fixed factors (nlme R package, Pinheiro et al., 2019). To investigate the effect of individual variables on geckos' body temperature, we fitted a mixed-effects model (Mod2) with body temperatures as the dependent variable, and the first-order interaction of body position (seven levels) and sex (two levels) as fixed effects. Individual identity was used as the random effect. In both models, body mass was used as a covariate to account for the potential effect of body size (i.e. thermal inertia) on body

temperatures. The statistical significance of single-level fixed factors was evaluated using ANOVA tables, while both random and fixed effects were evaluated using likelihood ratio tests (LRT) and by examining the summary tables of the models (*t*-value). Post hoc tests on multilevel factors were carried on using the lsmeans R package (Lenth, 2016).

Given the non-linearity of body temperature data (Shapiro-Wilkinson test = 0.9, $P < 0.0001$) and the repeated measure design of the experiment, we analysed the relationship between cloacal temperatures (contact thermometer) and the temperatures of other body parts (infrared camera) following the method of Barroso et al. (2016). Hence, the relationship between the cloacal temperature and each of the other body parts temperatures was investigated using OLS with resampling (lmodel2 R package, Legendre 2018, 999 resampling). Calibration parameters and functions values were extracted to compare the relationship between cloacal temperatures and the other body parts' temperatures.

Finally, in order to better represent the relationships between the different body temperatures and to allow comparisons between geckos and lacertids, Spearman correlation and Multidimensional Scaling (vegan R package, Oknasen 2019) were performed for *T. mauritanica*'s temperatures. The same analyses were also applied to the data from Barroso et al. (2016) for lacertids and compared with the results obtained for this study. All analyses were performed using the R software, version 3.6.0 (R Core Team, https://www.r-project.org).

2.4. Results

Regarding the potential effects of procedures on body temperature (Mod1), we found significant differences in body temperatures depending on the trial (chi-squared = 286.2, $P < 0.0001$), while the heating status was not significant ($P > 0.05$). More specifically, post hoc tests with Tukey correction for multiple comparisons showed that average body temperatures significantly differed between the first trial and all others, and between the last trial and all others (Supplementary Table S2.1), showing that the first and last trial tended to underestimate geckos' body temperature.

In the second model, in which we tested for the effect of individual variables (sex, mass and body position) on body temperature, we found a significant contribution of individual variability, as expressed by the random effect (LRT = 502.4, $P < 0.001$). Contrarily, body

mass was not significant (P = 0.42), despite the fact that males were heavier than females (mean M = 7.587 g, mean F = 5.769 g; Mann-Whitney test = 35455, P < 0.0001).

There was also no significant difference between sexes (P = 0.66; Fig. 2.2) in body temperature for any of the body parts considered (mean ± SD cloacal temperature M = 22.9 ± 5.01 ºC; F = 22.4 ± 4.80 ºC; see Table 2.1 for mean temperatures of remaining body parts and Fig. 2.2 for graphical representation).

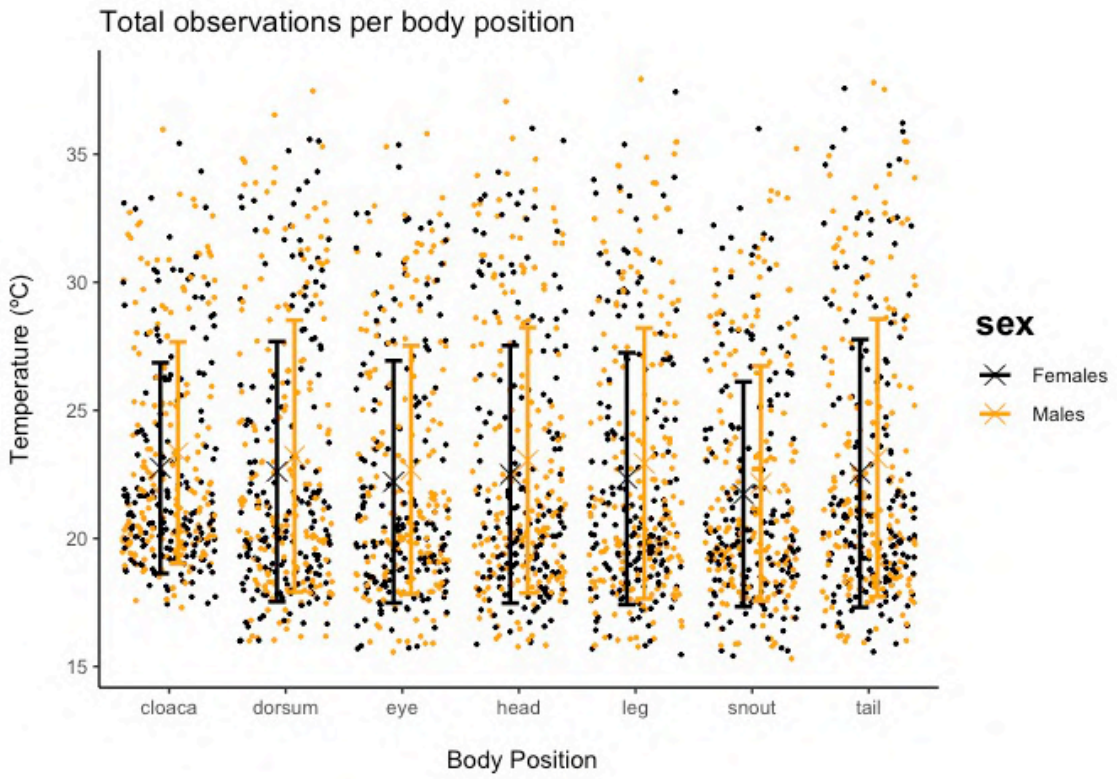

Fig. 2.2 - *Graph showing the scatter of the temperatures obtained for each body position for both males and females. The mean and standard deviation of the temperature of each body part are also shown further emphasizing the reported similarity between the sexes as well as between the different body parts (i.e. low regional heterothermy).*

Body temperature significantly differed depending on the position considered (chi-squared = 16.27, P = 0.012). However, statistically significant differences were detected only between the snout and cloaca (*t*-ratio = 3.446, P = 0.0104) and between the snout and dorsum (*t*-ratio = 3.016, P = 0.041).

Nonetheless, when comparing regressions between cloaca and the other body parts, the leg provided the best R^2 (0.97) (Table 2.1 and Fig. 2.3) which also showed the lesser line angle with cloacal temperature (0.84º). Slope values tended to increase from snout to tail, while intercept was less predictable (Table 2.1, Fig. 2.3). The best (i.e. closer to

zero) average intercept was provided by the eye which, however, showed also the second widest 95% confidence interval for this parameter (Table 2.1). Similarly, the eye also provided the best (i.e. closer to 1) slope values (Table 2.1), but again was affected by the second greatest variability (Table 2.1, Fig. 2.3). Nonetheless, despite the comparatively wide 95% CIs of the eye's slope and intercept, a strong association (R^2 = 0.924) for this regression was still obtained.

Fig. 2.3 Graph	Body Part	R^2	Slope	Slope 95% CI	Intercept	Inter. 95% CI	Temperature Mean ± SD (ºC)
A	Eye	0.924	**1.087**	1.054 1.120	**-2.689**	-3.462 -1.917	M: 22.71 ± 4.83 F: 22.22 ± 4.73
B	Snout	0.871	0.879	0.844 0.915	3.817	3.022 4.611	M: 22.16 ± 4.58 F: 21.77 ± 4.4
C	Head	0.948	1.176	1.147 1.205	-4.351	-5.026 -3.677	M: 23.11 ± 5.17 F: 22.6 ± 5.05
D	Dorsum	0.964	1.208	1.184 1.232	-4.944	-5.515 -4.374	M: 23.27 ± 5.29 F: 22.72 ± 5.12
E	Leg	**0.970**	1.242	1.219 1.265	-5.827	-6.361 -5.293	M: 22.99 ± 5.26 F: 22.45 ± 4.95
F	Tail	0.940	1.172	1.141 1.203	-4.384	-5.111 -3.658	M: 23.17 ± 5.42 F: 22.65 ± 5.25

Table 2.1 *- Results of the OLS regressions between cloacal and the other body parts of* Tarentola mauritanica*. Bold values indicate the body position that provided the best fit (highest R^2; slope closer to 1; intercept closer to 0). Underlined values indicate the narrowest 95% confidence intervals. A column with the mean ± SD temperature was also included to emphasize the similarity of average temperatures between sexes as well as between body parts. For reference and ease of comparison, the mean ± SD of cloacal temperatures of male and female geckos were 22.9 ± 5.01 ◦C and 22.4 ± 4.80 ◦C respectively.*

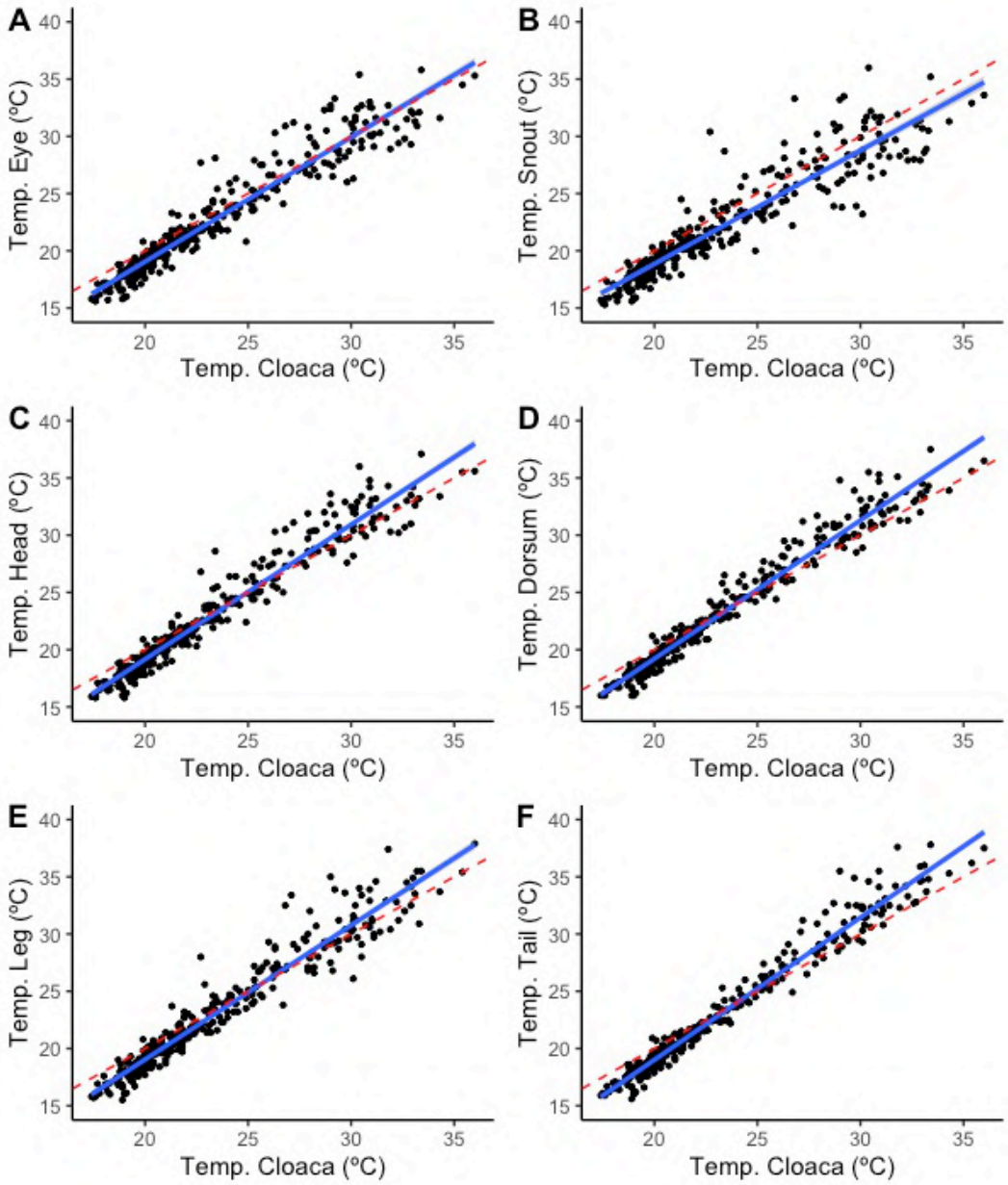

Fig. 2.3 *- Graphical representation of the OLS regression lines (blue) and corresponding 95% confidence intervals (grey shading - very close to the blue lines given the high fits of the models) between the thermography-measured body temperatures (y-axes) and the thermometry-measured cloacal temperature (x-axes). The dotted red lines*

Results from the OLS regression and from the correlation analysis for *T. mauritanica* showed strong association between all measured variables (Table 2.1, Fig. 2.3 and Supplementary Table S2.2), with the lowest value having an R^2 of 0.871 and a correlation score of 0.956 (Table 2.1 and Supplementary Table S2.2). Interestingly, both the correlation analysis and MDS plots were congruent with cloacal temperature as the most different measurement and with all the other temperature measurements clustered

together (Fig. 2.4). However, in the correlation analysis head and dorsum were clustered the closest, followed by tail and leg, with eye and snout forming another cluster, whilst in the MDS (stress value = 0.1144) dorsum was closer to tail and to similar distances from leg and head (Fig. 2.4). Remarkably, eye and snout, respectively, were the most different measurements within that cluster.

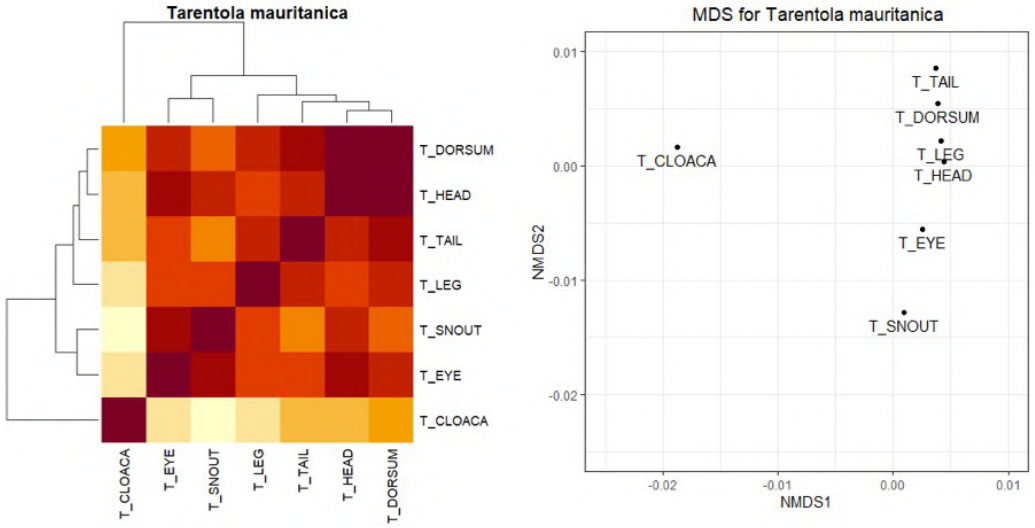

Fig. 2.4 - *Correlation heatmap and MDS plot (stress value = 0.114) for the T. mauritanica dataset. In both plots a similar pattern is observed, with cloacal temperature as the most different and all other measurements clustering together (T_DORSUM – Dorsum's temperature; T_HEAD – Head's temperature; T_TAIL – Tail's temperature; T_LEG – Leg's temperature; T_SNOUT – Snout's temperature; T_EYE – Eye's temperature; T_CLOACA – Cloaca's temperature).*

On the other hand, data on three lacertid species from Barroso et al. (2016), here subjected to Spearman correlation and MDS analyses, showed a different pattern from *T. mauritanica*. Namely, while cloacal temperature was the most differentiated measurement for both methods, the rest of measurements showed more heterogeneity in the lacertids (Fig. 2.5). In the correlation analysis, all measured variables were similar, with strong correlation scores (Supplementary Tables S3 to S5). However, eye and especially snout showed consistently lower scores than the rest. The difference is only slight and the correlation scores are high nonetheless (>0.87), hence the biological significance of this difference is arguable especially in light of the OLS results reported by Barroso et al. (2016).

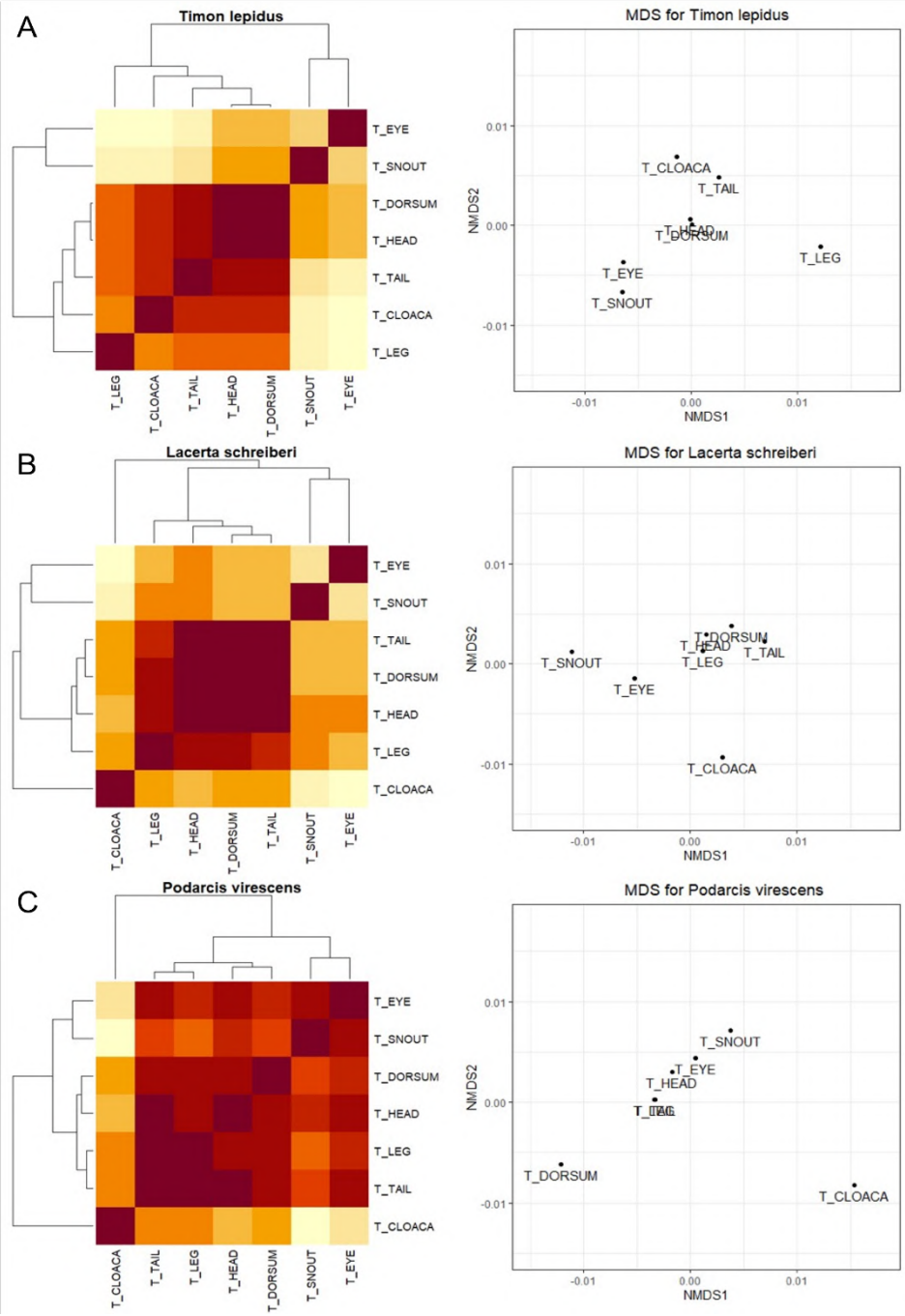

Fig. 2.5 - *Correlation heat maps and MDS plots for (A)* Timon Lepidus *(stress value = 0.083) showing the measured variables clustering in two main groups: the first one with temperature measured in the eye and in the snout and the second one with dorsum, head, tail, cloaca and leg, from more to less correlated. Correlation values were high, ranging from 0.893 to 0.998. (T_DORSUM – Dorsum's temperature; T_HEAD – Head's temperature; T_TAIL – Tail's temperature; T_LEG – Leg's temperature; T_SNOUT – Snout's temperature; T_EYE – Eye's temperature; T_CLOACA – Cloaca's temperature), (B)* Lacerta schreiberi *(stress value = 0.096) showing cloacal temperature as the most different measurement. Other variables were clustered in two different groups, with snout and eye in one side and leg, head, dorsum and tail on the other. All measured variables were highly correlated, with the lowest correlation value being 0.935. (T_DORSUM – Dorsum's temperature; T_HEAD – Head's temperature; T_TAIL – Tail's temperature; T_LEG – Leg's temperature; T_SNOUT – Snout's temperature; T_EYE – Eye's temperature; T_CLOACA – Cloaca's temperature) and* Podarcis virescens *(C) (stress value = 0.047) all variables were highly correlated, with cloacal temperature as the most different measurement but still scoring a value of 0.873 as its lowest correlation value. The variables clustered in two main groups, with snout and eye on one side and tail, leg, head and dorsum on the other.*

2.5. Discussion:

An increasing number of studies now acknowledge the need to validate the use of infrared thermography to measure body temperature of reptiles (Barroso et al., 2016; Luna et al., 2013; Sannolo et al., 2014). Nonetheless, most are focused on phylogenetically closely related lizards (e.g. belonging to the Family Lacertidae), with similar body shape and anatomy, physiology and ecology. Hence, devising a generalized validation of this tool for all lizards, from a small subset of species, is unreliable at best. Thus, the need has arisen to test thermography over a broader range of squamate groups moving towards a unified standard methodology for the use of thermography to measure body temperature of reptiles.

The present study found very strong correlations (OLS regression $R^2 > 0.87$, Spearman Correlation > 0.95) between the cloacal temperature and the thermography-measured temperatures of several body locations in *T. mauritanica*, as also described for lacertids in Barroso et al. (2016). In fact, this study found stronger correlations than those obtained for the lacertids, data of which were also analysed further in this study (Supplementary Tables S2.2 to S2.5) (OLS regression $R^2 > 0.83$, Spearman Correlation Scores > 0.91).

Furthermore, when the R^2 of the OLS models are compared between those of *T. mauritanica* to the more closely sized lacertid, *Podarcis virescens,* in Barroso et al. (2016), the increased strength of correlations found here becomes even more obvious. The similar size and mass of these two species should mean a similar degree of thermal inertia as well as a similar distance between the different body parts tested. This result is, nonetheless, contrary to what could be expected based on the behaviour and ecophysiology of these species. *Tarentola mauritanica*'s apparently more flexible approach at thermoregulation, perhaps due to its diurnal, and crepuscular habits, would indicate a lesser concern towards very precise control of body temperatures. This, in theory, results in less evident regional heterothermy leading to less consistent and more unpredictable body temperature gradients (Webb et al., 1972). The latter should have produced weaker correlations between temperatures of different body parts of *T. mauritanica*, contrary to what is found here.

For the previously outlined reasons, regional heterothermy in *T. mauritanica* should be less marked than what was observed in *P. virescens*. This pattern was clear when observing a specimen through a thermal camera (Fig. 2.1). This is further supported by the strong correlations found between the temperatures of all body parts (Supplementary Table S2.2).

Perhaps *T. mauritanica* does in fact thermoregulate precisely but dedicates less resources to maintaining body temperature gradients, aided by its low thermal inertia. A different experimental set up would be necessary to test this. Alternatively, the morphological and physiological mechanisms responsible for maintaining body temperature gradients, found in many other lizard groups, may simply be absent or less prominent in geckos (Webb et al*.,* 1972). Many of these mechanisms, such as heart rate hysteresis and peripheral circulation controls, are widely studied among lacertids, agamids and iguanids (Bruner, 1907; Heath, 1966; Porter and Witmer, 2015) yet there is a lack of literature regarding these conditions in geckos (Bauer, A., pers. com.) (but see Grimmond et al., 1981; and Webb et al., 1972).

Most of such phenomena rely, to some extent, in adaptations of the circulatory system. The work of Mahendra (1942) on the anatomy of the gecko *Hemidactylus flaviviridis* (Family Gekkonidae) hypothesizes about geckos having a more basal form of circulatory system among squamates (but see Porter and Witmer, 2020). If also applicable to *Tarentola*, this could explain the apparent lack of precise control over surface body temperature gradients observed. However, the otherwise extensive study lacks a description of the cephalic and peripheral circulation, such as what is available for other groups as lacertids (Bruner, 1907) and iguanids (Porter and Witmer, 2015). Blood circulation would be a major factor influencing the control of regional heterothermy (Heath, 1966; Webb et al*.,* 1972), along with thermal inertia. Furthermore, other studies have also shown the presence of complex vascularization patterns in the feet of geckos (Russel, 1981) which may prove relevant for the control of thigmothermic heat transfer.

Despite the strong correlations observed between temperatures of all body parts, the MDS plots and the correlation matrices of the *T. mauritanica* clusters the surface temperatures together and the cloacal temperature appears as an outgroup (Fig. 2.4). The same is partially true for the different lacertid species, with the exception of *Timon lepidus*, the largest species tested (Fig. 2.5). Furthermore, for *T. mauritanica* the observed arrangement of relationships follows a geographic pattern with the physically closer points having more similar temperatures amongst themselves than to more distant body parts. This non-arbitrary pattern of temperature gradients does support the presence of some regional temperature control, albeit the very small absolute differences observed in *T. mauritanica* (Fig. 2.2; Table 2.1) would suggest this control may not be very precise.

At a broader scale, this study found that the first and last trials of each experimental set significantly lower body temperatures than the others. However, caution must be taken

to allow for sufficient time for the animal to acclimate to the experimental set up as this may have been the cause for the lower temperatures observed in trial 1. Equally, attention must also be paid not to overexpose the animals to very long periods in the gradients as they may become stressed (e.g. over handling, dehydration, too much energy spent thermoregulating) and decrease their thermoregulatory precision or set point, which could explain the lower temperatures observed in trial 6.

In the case of *T. mauritanica*, the relatively large size of its eyes, compared to that of other lizards, should have made this structure a relatively easy one to target in IR photos. However, this was not always the case. Likely due to the presence of a scale covering the eye - the brille - a structure common to most geckos (Bellairs, 2009), eyes were often isothermal with the surrounding area and thus difficult to distinguish. Furthermore, we were unable to find any literature on the detailed vascularisation of gecko's cephalic region, thus we are not certain how well the eye is vascularised and whether there is a large storage of blood (i.e. sinus orbitalis), and thus of temperature, coming from the brain, as is common in many other squamate groups (Bruner, 1907; Porter and Witmer 2015). Further research should dedicate some effort towards describing the pattern of cephalic circulation of geckos and its importance to thermoregulation as well as to investigate whether the presence of the brille or the fact that geckos frequently lick their eyes (Bustard, 1963) would significantly affect heat exchange with the environment (e.g. through evaporative cooling).

Ultimately, the results obtained show that body surface temperatures of *T. mauritanica* measured through thermography can reliably be used to infer internal body temperature. The minute differences in temperatures between all body parts (Fig. 2.2) and the high R^2 values obtained by all the OLS regressions (Table 2.1), suggest that any body part could be used to infer internal body temperature with equivalent results, given the appropriate correction is applied. Nonetheless when also considering the intercept and degree of slope of the models (Table 2.1, Fig. 2.3), to account for the presence of biases in the subsequent inferences, as well as the results obtained by Barroso et al. (2016), then the eye presents as the most ubiquitously suitable candidate body location to infer internal temperature.

2.6. Conclusion:

From this study we further emphasize the validity of IR thermography as a tool to obtain high-resolution temperature data which, given the appropriate calibrations, may still provide comparable results to those obtained by traditional, more invasive methods. Hence, we suggest this tool will prove increasingly useful in the field thermal ecology of ectotherms.

Furthermore, this study has also shown that, through a simple set-up and randomization of heating treatment, it is relatively simple and fast to obtain a sample of temperature data, over a representative thermal range, which can then be used to calibrate the tool for the species of interest. Further studies should focus on testing this tool over a wider variety of squamates, particularly in those groups with larger body size ranges (e.g. varanids) and/ or with larger distances between the eye and the cloaca (e.g. snakes) in order to fully validate the proposed methodology. Furthermore, future work is needed to determine the importance of evaporative cooling from the chosen body part and its subsequent effect on the temperature readings while some efforts should also be diverted towards establishing appropriate emissivity values for the tissues from which readings are taken (e.g. skin types, osteoderms, eye, etc).

Ultimately, this study provides further support towards establishing the eye as the standard location to infer internal body temperature of lizards from thermography measured temperatures, as previously suggested by Barroso et al. (2016). Nonetheless it is still important to consider all the limitations revised in Barroso et al. (2016) including the pixel size effect (Faye et al., 2016), the distance between the camera and the subject (Faye et al., 2016), the need for species-specific calibrations, the need to establish appropriate emissivity values, the effect of evaporative cooling (particularly in the eye and snout), etc. when considering the use of IR cameras to infer internal body temperatures of lizards.

2.7. Acknowledgements

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2.9. Supplementary Materials:

contrast	estimate	SE	df	t.ratio	p.value
1 - 2	-3.803	0.306	2417	-12.427	**< .0001**
1 - 3	-3.083	0.306	2417	-10.090	**< .0001**
1 - 4	-3.471	0.305	2417	-11.375	**< .0001**
1 - 5	-3.687	0.315	2417	-11.688	**< .0001**
1 - 6	-4.762	0.307	2417	-15.527	**< .0001**
2 - 3	0.721	0.286	2417	2.523	0.1179
2 - 4	0.332	0.285	2417	1.167	0.8524
2 - 5	0.116	0.293	2417	0.395	0.9988
2 - 6	-0.959	0.287	2417	-3.339	**0.0110**
3 - 4	-0.388	0.284	2417	-1.366	0.7473
3 - 5	-0.605	0.293	2417	-2.061	0.3084
3 - 6	-1.679	0.286	2417	-5.870	**< .0001**
4 - 5	-0.216	0.292	2417	-0.742	0.9767
4 - 6	-1.291	0.286	2417	-4.518	**0.0001**
5 - 6	-1.075	0.296	2417	-3.630	**0.0039**

Table S 2.1 *- Summary output table of the Tukey Post hoc pairwise comparisons between trials (1 to 6).* ***Bold*** *values indicate where statistically significant differences were found.*

P value adjustment: tukey method for comparing a family of 6 estimates

	Cloaca	Eye	Snout	Head	Dorsum	Leg
Eye	0.964					
Snout	0.956	0.993				
Head	0.972	0.995	0.989			
Dorsum	0.976	0.991	0.984	0.997		
Leg	0.967	0.989	0.987	0.988	0.989	
Tail	0.974	0.988	0.981	0.992	0.994	0.991

Table S 2.2 *- Spearman correlation values between all the measured temperatures in* T. mauritanica *ranging from 0.956 to 0.995. All IR camera measurements were highly correlated (>0.98).*

	Cloaca	Eye	Snout	Head	Dorsum	Leg
Eye	0.893					
Snout	0.904	0.925				
Head	0.975	0.932	0.939			
Dorsum	0.975	0.930	0.939	0.998		
Leg	0.948	0.895	0.91	0.961	0.959	
Tail	0.978	0.908	0.915	0.987	0.987	0.96

Table S 2.3 *- Spearman correlation values for all the measured temperatures in* Timon lepidus *ranging from 0.893 to 0.998.*

	Cloaca	Eye	Snout	Head	Dorsum	Leg
Eye	0.935					
Snout	0.941	0.95				
Head	0.962	0.97	0.968			
Dorsum	0.962	0.961	0.962	0.997		
Leg	0.966	0.961	0.971	0.99	0.989	
Tail	0.965	0.957	0.958	0.995	0.996	0.987

Table S 2.4 *- Spearman correlation values for all the measured temperatures in* Lacerta schreiberi *ranging from 0.935 to 0.997.*

	Cloaca	Eye	Snout	Head	Dorsum	Leg
Eye	0.901					
Snout	0.873	0.982				
Head	0.923	0.988	0.973			
Dorsum	0.929	0.978	0.958	0.986		
Leg	0.939	0.971	0.955	0.982	0.98	
Tail	0.942	0.979	0.959	0.989	0.985	0.994

Table S 2.5 *- Spearman correlation values for all the measured temperatures in* Podarcis virescens*, ranging from 0.873 to 0.994.*

3. Chapter 3 - Novel method to investigate thermal exchange rates in small, terrestrial ectotherms: A proof-of-concept on the gecko *Tarentola mauritanica*

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3.1 Abstract

Thermoregulating ectotherms may resort to different external heat sources to modulate their body temperature through an array of behavioural and physiological adaptations which modulate heat exchange with the environment and its distribution across the animal's body. Even small-bodied animals are capable of fine control over such rates and the subsequent re-allocation of heat across the body. Such thermal exchanges with the environment usually happen through two non-mutually exclusive modes: heliothermy (radiant heat gain from the sun) or thigmothermy (heat gained or lost via conduction). Classically, the study of these phenomena has relied on invasive methodologies which often disregard the effect of stress, behaviour and regional heterothermy on the rates and patterns of thermal exchange across the body of the animal. This study proposes a novel experimental methodology, capitalising on thermography, to provide an alternative method to less invasively obtain reliable body temperatures of thermoregulating ectotherms, while allowing behaviour and heating mode to be considered when quantifying thermal exchange rates. This methodology was tested in the gecko *Tarentola mauritanica,* where twenty males were allowed to heat up and cool down under a novel experimental set-up which isolates heliothermic and thigmothermic processes, while being recorded with a thermal camera. The study revealed differences in the heating and cooling rates of several body parts per treatment suggesting that thermal exchanges are complex even in small ectotherms. Ultimately, the described set-up provides the opportunity to revisit classical questions with a less invasive and more flexible experimental approach, enabling heliothermic and thigmothermic processes to be disentangled. The described methodology also better integrates behaviour and physiology while obtaining higher temporal and spatial resolution of body temperatures in a thermoregulating ectotherm.

3.2. Introduction

Thermoregulating ectotherms, such as many reptiles, must rely on a combination of external heat sources, behavioural strategies and physiological mechanisms to modulate body temperature within an optimal range (Angilletta et al., 2006; Belliure & Carrascal, 2002; Huey & Slatkin, 1976). This approach allows reptiles to occupy a wide range of environments, from tropical forests and deserts to temperate regions and even subarctic areas (Taylor et al., 2021; Vitt & Caldwell, 2014). Compared to other vertebrates, reptiles highlight the role of behavioural thermoregulation for maintaining, and even increasing, a species distribution and diversity from what is expected given their physiological critical limits (Bodensteiner et al., 2021; Camacho et al., 2024; Huey et al., 2012). Indeed, behavioural thermoregulation in reptiles has been recognized as a pivotal element in their biology, ecology and physiology (e.g. Angilletta, 2009; Goller, Goller, & French, 2014), allowing them to attain body temperatures close (or equal) to the thermal optima maintaining a range of physiological processes, without the costly energetic needs associated to endothermy (Angilletta, Niewiarowski, & Navas, 2002; Sears & Angilletta, 2015).

Heat exchange between the environment and an organism has been deeply studied since the 1950's aiming to understand, often from a theoretical point of view, the thermodynamic processes governing the mechanistic interplay between an animal's thermal state and its surroundings (e.g. Bakken & Gates, 1975; Porter & Gates, 1969). In essence, these thermal exchanges occur through a combination of non-mutually exclusive radiative, convective, conductive, and evaporative processes (Porter & Gates, 1969; Seebacher & Franklin, 2005) (explained by Birkebak, 1966) on which a thermoregulating animal may exert some control, via physiology and/or behaviour, to optimise its thermal exchanges.

For ectotherms in temperate regions, convection is expected to provide a lesser contribution towards the increase in body temperature yet, along with evaporation (DeNardo, Zubal, & Hoffman, 2004; Loughran & Wolf, 2023), may be an important mechanism of heat loss when an organism operates close to the upper limits of their thermal range (Cabello-Vergel et al., 2021; Moss et al., 2019; O'Connor et al., 2018; Silanikove, 2000). Moreover, dehydration tends to restrict temperate reptiles to body temperatures lower than their optima, when water is not available for evaporative cooling mechanisms to be engaged (Sannolo & Carretero, 2019).

For cooling, however, and particularly in cold or windy environments, convection usually underpins an inherent, and often counter-productive (Maia-Carneiro, Dorigo, & Rocha, 2017; Ortega, Mencía, & Pérez-Mellado, 2017; but see Gontijo et al., 2018), loss of body temperature (Bogert, 1949; Spears et al., 2024), which may represent significant costs to the temperate ectotherm. These costs may include restricted activity periods, increased time spent thermoregulating and increased risk of dehydration (Maia-Carneiro, Dorigo, & Rocha, 2017; Ortega, Mencía, & Pérez-Mellado, 2017; Virens & Cree, 2022). Hence, ectotherms from such environments often show a range of behavioural and physiological adaptations to minimise the rates of disadvantageous heat loss from convection and maximise the rates of heat gain, usually from radiation and/or conduction (Barton, Porter, & Kearney, 2014; Tattersall, Cadena, & Skinner, 2006).

Finally, radiation and conduction are the two most effective and ubiquitous ways that reptiles utilise to modulate their body temperature towards a preferred set-point (Garrick, 2008). The active process of regulating body temperature from solar radiation (i.e. heliothermy) is a prominent strategy used to warm up and has allowed high levels of diversification in several reptile groups from a range of environments (e.g. Garcia-Porta et al., 2019). On the other hand, the active exchange of heat through direct contact with the substrate (i.e. thigmothermy) is less ubiquitously reported (see Ortega, Mencía, & Peréz-Mallado, 2017; Ortega et al., 2019) and has frequently been associated with species with limited access to solar radiation, such as nocturnal, forest or fossorial animals (Garrick, 2008). However, it may also represent a complement to heliothermy, even in primarily heliothermic species (Carrascal et al.,1992). Notably, heliothermy (i.e. heating up by IR radiation from the sun) is usually a unidirectional process since the initial heat supply will only act as a source but not as a sink. Nonetheless, it is relevant to point out that any hot body – including the animal – becomes a radiant heat source itself, as it inevitably emits IR radiation to its surroundings, hence effectively cooling down by means of radiation (Bakken & Gates,1974; Tracy,1976).

Thigmothermy, however, is inherently a more bidirectional phenomenon whereby the direction and rate of heat transfer depend respectively on the direction and magnitude of the temperature differential between the animal and its substrate (Belliure & Carrascal, 2002). Hence, the animal can actively select a substrate either warmer or colder than itself to act as a heat source or sink. Thus, thigmothermy can aid in the fine tuning of an animal's body temperature and its distribution across the body, even while heating heliothermically (Belliure & Carrascal, 2002). This suggests that, although less studied experimentally (see Ortega, Mencía, & Peréz-Mallado, 2017; Ortega et al., 2019),

thigmothermy may be more common than often considered. For example, it is the main, but not exclusive, thermoregulatory strategy in the highly diverse group of Gekkota (Garrick, 2008).

Ectotherm thermal ecology studies abound with a range of tools and methods used to acquire body temperature of the animals, both in field and laboratory contexts - extensively reviewed by Camacho & Rusch (Camacho & Rusch, 2017), and Taylor et al. (Taylor et al., 2021). In this field, there is an increasing trend to use more powerful but less invasive temperature measurement tools such as infrared (IR) thermometers (e.g. Chukwuka, Virens, & Cree, 2019; Cox et al., 2023), dataloggers (e.g. Virens & Cree, 2022) and thermal cameras (e.g. Sannolo et al., 2014; Barroso et al., 2016; Barroso et al., 2020). This, in turn, is also allowing for more integrative methodologies (e.g. Hastings et al., 2023; Spears et al., 2024). Notwithstanding, although such tools are increasingly common in research on ectotherm thermal preference, tolerance or performance, they are still uncommon in studies evaluating thermal exchange rates of these animals (see Rutschmann et al., 2020).

Historically, many of such studies on thermal exchange rates involved restraining the animal under a heat source while measuring its body temperature change at given time intervals, usually via a cloacal temperature probe (e.g. Bartholomew & Tucker, 1963; Bartholomew & Tucker, 1964; Claussen & Art, 1981; Díaz, Bauwens, & Asensio, 1996; González-Morales et al., 2021). These focused on the physiological control of heating rates, disregarding the effect of behaviour (such as posturing), and imposing high stress levels, while neglecting differential heat distribution patterns to body parts other than the cloaca. Only a few studies opted for less manipulative approaches where animals were allowed to freely thermoregulate in a terrarium with only the desired heating mode (heliothermic or thigmothermic heat source) available throughout (e.g. Belliure & Carrascal, 2002). While the latter scheme allowed for more natural thermoregulatory behaviour and physiology, this less invasive approach inevitably came at the cost of measuring temperature of the animals less often as well as in a single body part (i.e. the cloaca). Hence, studies addressing this trade-off and allowing to measure temperature less-invasively, continuously and in multiple body parts, may extend such findings to spatial and temporal patterns of heat gain.

Meanwhile, the use of thermography has been rising in thermal ecology and physiology studies (Playà-Montmany & Tattersall, 2021; Spears et al., 2024; Tattersall, 2016). Often, radiometric photos of a specific point in time are used to measure the body temperature of one or more body parts (e.g. Barroso et al., 2016; Barroso et al., 2020). This

constitutes an improvement on previous methodologies (but see Zhang et al., 2008), which either relied on more invasive techniques (e.g. cloacal measurements) (Bartholomew & Tucker, 1963; Bartholomew & Tucker, 1964; Claussen & Art, 1981; Díaz, Bauwens, & Asensio, 1996; González-Morales et al., 2021) or failed at attaining a short-term continuum of measurements (Belliure & Carrascal, 2002). Nevertheless, thermography-based studies have so far also mostly disregarded the integration of behavioural data and continuous, non-invasive temperature measurements (but see Hastings et al., 2023 and Rutschmann et al., 2020). Ultimately, this hindered the monitoring of fast physiological processes, such as maximum rates of thermal transfer and dynamic patterns of regional heterothermy, under behaviourally thermoregulating animals.

Here we provide and implement an alternative methodology that relies on radiometric video to investigate fast thermal exchange processes in small lizards, with the main aim of acquiring localised heat exchange rates of different body parts. Furthermore, we present and test a novel and flexible set-up to heat up or cool down an animal while controlling, or isolating, for the mode of heat exchange (heliothermic vs thigmothermic). We selected the crepuscular gecko *Tarentola mauritanica* (Linnaeus, 1758) as a model species due to its behavioural and physiological adaptations for obtaining heat through both thigmothermy and heliothermy. Ultimately, this method allows exploring differences in heat exchange patterns of different body parts, depending on the thermoregulatory strategy of interest (heliothermy vs thigmothermy), while accounting for both physiological and behavioural variables, such as the ability to regulate blood flow across the body and to adjust body posturing, respectively.

3.3. Materials and Methods

3.3.1 Study species

The common wall gecko, *Tarentola mauritanica* (Linnaeus, 1758), is a mid-sized (mean SVL = 70mm, mean mass = 9g) temperate lizard species with a circummediterranean distribution (Vogrin et al., 2017). Although it can be found basking, capitalising on both heliothermy and thigmothermy, especially during the morning or late afternoon (Rato & Carretero, 2015), most of its activity occurs at night when it is expected to use the thermal inertia of its substrate to warm up thigmothermically. This bimodal thermoregulation pattern makes this species an interesting model to study the physiology of thermal exchange rates.

3.3.2. Sampling and husbandry

Animals were collected during spring 2019 from four different populations: Évora (coordinates: 38.532, -8.018), Torres Vedras (39.087, -9.246) and Portimão (37.197, -8.539) in Portugal, and Ayamonte (37.260, -7.347) in Spain. The geckos were attracted by a laser pointer on vertical surfaces (Cole, 2004) and then caught using a slipknot (García-Muñoz & Sillero, 2010) or by hand. The animals were housed in 50Lx30Wx25H cm terraria with stacked rocks as refugia and a water dish for hydration. Kept in an air-conditioned room at 19°C, animals were provided heat from dusk until dawn (19:00h to 07:00h) through an array of 150W infrared light bulbs. Natural photoperiod was maintained through a diffuse glass window. Food consisting of house crickets (*Acheta domesticus*) or mealworm larvae (*Tenebrio molitor*) was provided three times a week.

Only adult males were used in the experiments to prevent any possible effects of ontogeny or of oogenesis (in females). To sex the animals, the base of the tail, adjacent to the cloaca, was gently squeezed to evert the hemipenes of males (Atzori et al., 2007). Five individuals per population were randomly chosen, totalling 20 animals with different body sizes. Since multiple populations were sampled and the size of *T. mauritanica* differs considerably between populations (Massetti et al., 2017), the resulting sample encompassed a typical overall size range for adult males of this species. This aided in accounting for the potential effect of size (and hence thermal inertia) in the rates of thermal exchange and patterns of heat distribution across the body. For each animal, snout-to-vent length (SVL) was measured to the nearest 0.01mm using digital callipers (Perel Digital Calliper 3472, precision: 0.01mm, accuracy: ±0.02mm) and mass with a precision balance (Sartorius M-Pact AX224, Sartorius AG, Goettingen, Germany, precision: 0.0001g). Mean SVL ± standard error (SE) for the tested individuals was 70.01 ± 1.76mm (SVL range: 55.21 – 82.17mm; Fig. 3.1) and mean mass ± SE was 8.87 ± 0.46g during the heliothermic trials, and 8.90 ± 0.45g during the thigmothermic trials (overall mass range: 6.37 – 12.09g; Fig. 3.1).

To account for any individual-related discrepancies in the physiological responses, each individual was subjected to the two trials, in a random order, where different heating strategies (heliothermy vs thigmothermy), and the subsequent cooling process, were independently tested.

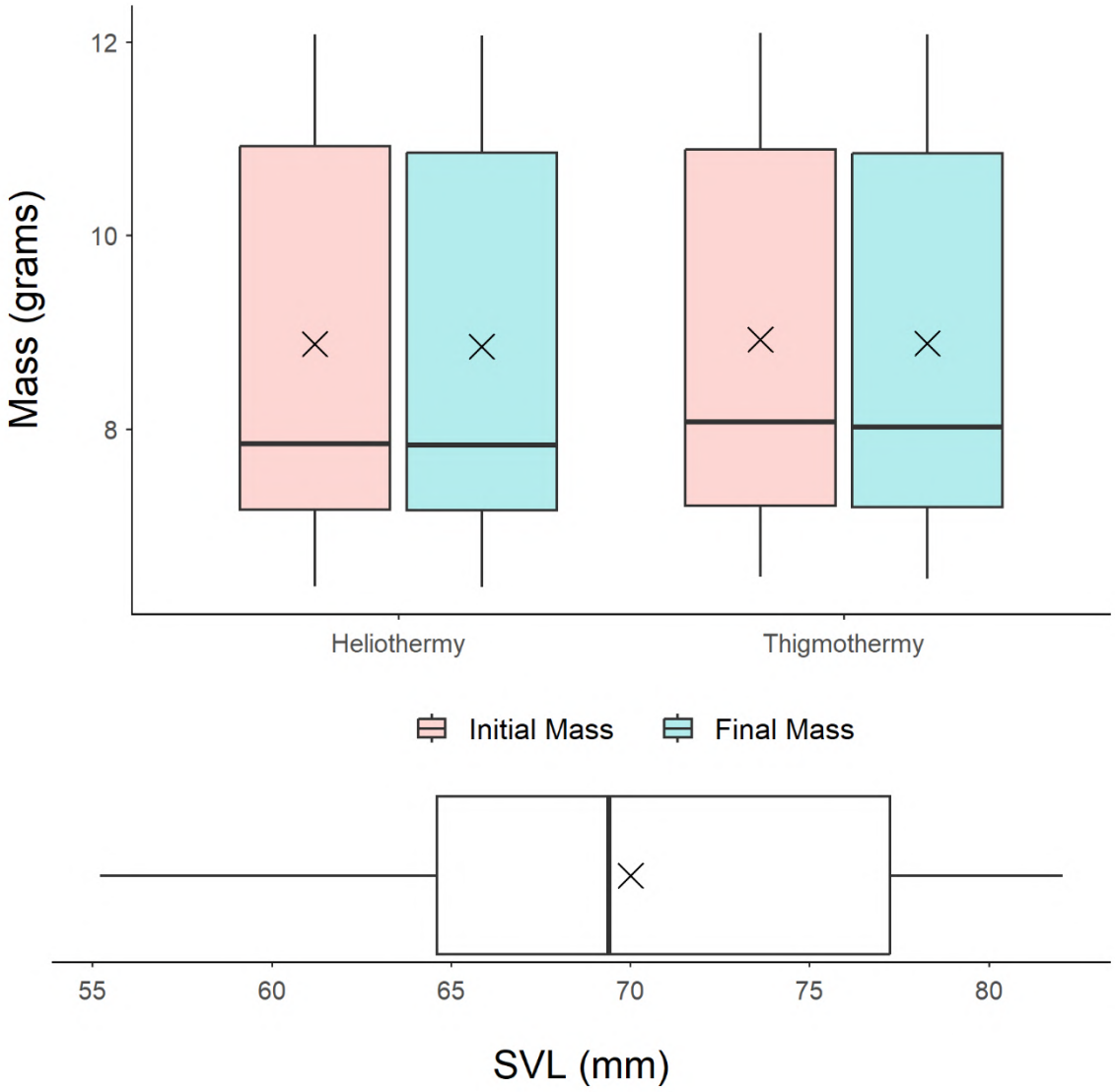

Fig. 3.1: Mass (at start vs at the end of each treatment trial) and overall Snout-to-Vent Length (SVL) of all the tested animals. The line in the middle of the boxplot represents the median value, while the "X" represents the mean value. No significant differences between initial and final mass were observed nor were there any differences in mass between treatments.

3.3.3. Experimental settings

In order to isolate heliothermy from thigmothermy, independent experimental arenas were devised. The set-up for simulating heliothermy (for simplicity, hereafter referred to as “heliothermy experiment”) consisted of a 110Lx50Wx30H cm aquarium partially filled with water, inside which a smaller (34Lx23Wx20H cm) aquarium was placed inside it, upside down and partially (5-10cm) emerging above the larger aquarium’s water level. The small, inverted aquarium was also filled with water, through vacuum suspension, resulting in a combined volume of water of circa 170 litres of water (Fig. 3.2). The bottom external face of the small aquarium’s surface served as the experimental arena on which the animals were positioned. Only glass aquariums were used, and the thickness of the glass wall kept constant (4mm), in order to ensure similar heat conductivity of the arena’s surface. A 150W infrared (IR) bulb was suspended 30cm above the arena’s surface. The water in both aquaria was monitored through a contact thermometer (Hibok 14, precision: 0.1ºC, accuracy: 0.3%) fitted with a k-type thermocouple probe inserted inside the small aquarium. This water was kept at 19.0 ± 0.5ºC by the thermal inertia of the large volume of the system and through periodic changes or top-ups with hot or cold water as needed. To avoid thermal gradients forming in the water column, pumps were used for circulation and mixing (total flow rate > 6500 l/h). During the heating part of the experiment, the IR bulb was kept on and then subsequently turned off for the cooling part of the trial (Fig. 3.2).

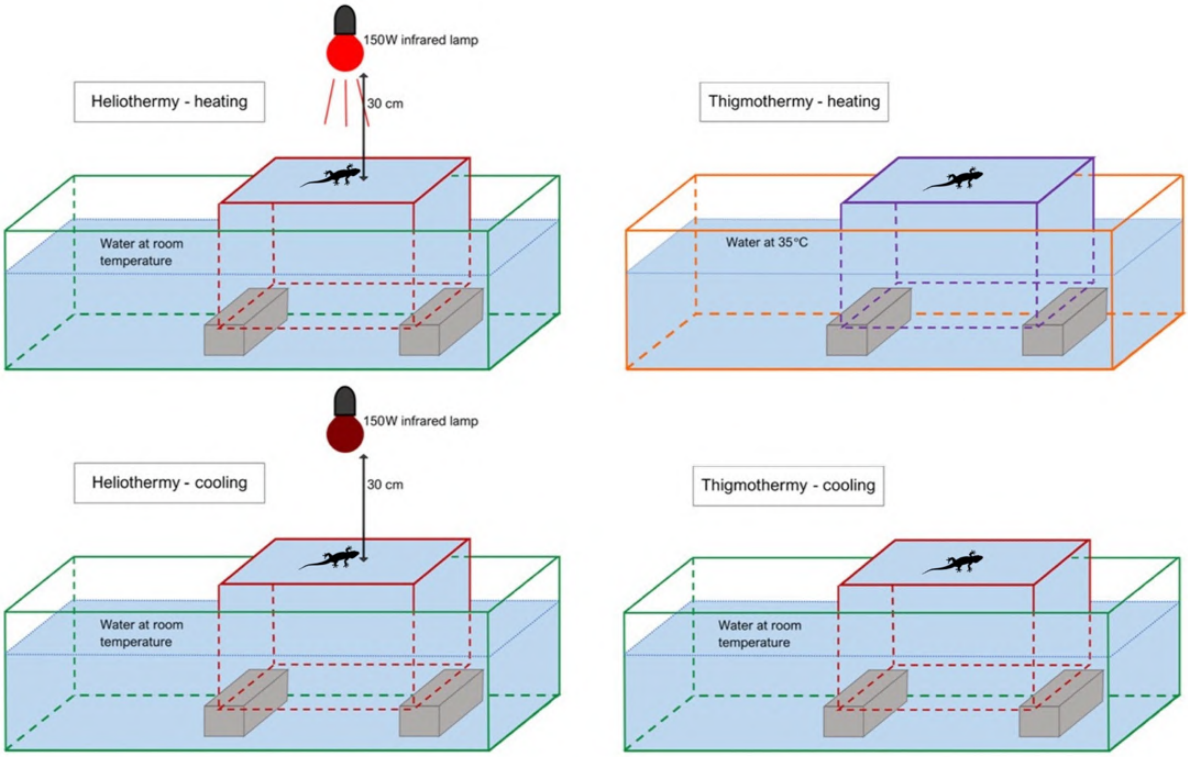

Fig. 3.2: Layout of the setup utilised for both heliothermy and thigmothermy experiments. An inverted, smaller aquarium is placed inside a larger one and filled with water by vacuum suspension. On the top, both different setups are in heating mode and at the bottom they are in cooling mode. Lizard silhouette marks the spot (external glass surface of the inverted, smaller aquaria) where the gecko was positioned during the experiment. Blue shading represents the water-filled volumes of the aquaria. Image credit: Valeria Marques

For the thigmothermy experiment, two separate experimental arenas were set up: one for the heating trial and another for the subsequent cooling trial. For heating, a setup similar to the one used for the heliothermy experiment (Fig. 3.2) was used. However, the water was previously heated and kept at 35.0 ± 0.5°C using aquarium heaters (EHEIM Thermocontrol 100W, adjustable temperature range: 18 - 34ºC, Accuracy: 0.5ºC, EHEIM GmbH & Co, KG, Germany), heat matts attached to the outer glass surface of the main tank and occasional top-ups with heated water. As in the heliothermic set-up, water temperature was monitored through the same k-type thermocouple probe inside the internal and inverted smaller aquarium and, as before, temperature gradients were avoided by the use of submerged pumps. The large volume of water and subsequent large thermal inertia facilitated the maintenance of a stable temperature.

For the cooling part of the thigmothermic experiment, immediately after the end of the heating trial, the animal was moved to a smaller replica of the experimental arena (a 60Lx30Wx28H cm aquarium with a 30Lx18Wx20H cm aquarium turned over inside resulting in a combined volume of water of circa 60 litres), with water at 19 ± 0.5 ºC (Fig. 3.2) - maintained as described for the heliothermic set-up. The differences in the dimensions of the aquaria used were solely due to availability of these. Nevertheless, future deployments of this methodology should aim to set-up exact replicas (i.e. same size, same total volume of water) of the arenas for both the heating and cooling trials.

The relocation from the thigmothermic heating arena to the respective cooling arena was fast (~2 seconds) by using a harness and leash, previously fitted to the animals to avoid touching them during the trial, which would interfere with their body temperature. Upon contact with the cooling arena, the video recordings and necessary additional measurements were immediately initiated synchronously (explained below).

For all the experiments, room temperature was controlled via an air conditioning unit set to 19.0 ± 0.5ºC and measured with a thermometer (Fluke 971 Temperature and Humidity Meter, precision: 0.1ºC, accuracy: ± 0.5ºC). Overall, air and water temperature were constant along the experiments, with mean air temperature of 18.83 ± 0.34 ºC, and water temperature of 18.82 ± 0.68 ºC for all the cooling and the heliothermic heating trials and 35.13 ± 0.26 ºC for the thigmothermic heating trials.

Prior to the experiment, each gecko was fitted with a harness and leash made of a light-weight, smooth dental floss, loosely tied around its waist. Caution was taken to ensure the harness was not impeding movement. Before being placed in the experimental arena, the geckos were left for a minimum of ten minutes to familiarise themselves with room temperature and the harness. The subject's movements were constrained to the centre of the experimental arena by the leash taped to the arena itself (around 15cm of range of motion) as well as through a 10cm tall cardboard visual barrier surrounding the arena. When needed, a brush was used to coax the animal back into the camera's field of view. Each animal was weighed on a precision scale (Sartorius M-Pact AX224, Sartorius AG, Goettingen, Germany, precision: 0.0001g) before and immediately after being tested. Although animals were fasted the day prior to testing, any defecation (often water) that took place between these measurements was noted.

When isothermal with the environment, the animal was placed in the test arena, described in Fig. 3.2 for the heliothermic heating experiment and thigmothermic heating experiment (in a random order, on separate days). Immediately upon contact with the

arena, a thermal video of the animal was recorded with an infrared (IR) thermal camera (FLIR T335, FOL 18 mm lens, sensitivity: < 0.05 °C; accuracy: ± 2%; IR image resolution: 320×240 pixels; IR video framerate: 10fps; FLIR Systems Inc., Wilsonville, Oregon, USA) fitted directly above the arena at a vertical distance of 30cm from its centre. This was used to monitor (during the experiment) and measure (*a posteriori*) the animal's body temperature. An additional CCTV RGB (closed circuit, colour video system MHD-CH30-130H, video framerate: 30fps) camera was set perpendicularly and at eye level to the animal, oriented towards the animal's flank, to monitor any escape attempts and to record the body posture of the animal under three different categories: elevated (just the feet in contact with the substrate), partially elevated (part of the ventral area in contact with the substrate) or flat (the animal laying the whole ventral surface flat on the substrate). The head and the tail were not considered in the posture categories.

The animal was allowed to heat up for a maximum of 10 minutes or until it reached a temperature of 35ºC at any body part (monitored through the IR camera's live feed). This is a conservative maximum threshold above the reported thermal preference for this species (i.e. 29.8 ± 3.79ºC (Vogrin et al., 2017). Hence, and since critical and voluntary thermal limits are not yet reported for *T. mauritanica*, this threshold was defined through a pilot study where signs of discomfort (e.g. restlessness, toepads curling up) where observed when foot temperatures exceeded 35ºC.

Immediately after the heating stage, the animal was then recorded cooling down for a maximum period of 10 minutes or until the temperature across the gecko's body was homogenous and isothermal with room temperature. Only one animal was tested at each time and multiple days were given between the heliothermic and thigmothermic trials of each individual.

During each trial, the following measurements were taken every 20 seconds, starting concurrently with the thermal video recording: water temperature inside the inverted aquarium (k-type thermocouple probe), air temperature (temperature and humidity metre), body posture (via the RGB video), whether the animal moved or stayed mostly stationary within the previous 20s period, and whether the animal defecated or not (regardless of whether it was water, urates or faeces) in that same period. Additionally, maximum body temperature of the animal was monitored through the thermal camera´s live feed to prevent the animal from reaching potentially stressful temperatures (conservative threshold set for 35ºC at any body part). A trial (heating or cooling) would end after ten consecutive minutes of thermal video recording or if any part of the animal reached the maximum temperature threshold. In the latter case, the trial would be

repeated if less than 3 minutes of thermal video had been attained. For this, and since such cases would only apply during the heating trials, the gecko was allowed to rest for at least 10 minutes, in order to return its body temperature to room temperature before repeating the process.

Data was always collected by the same person (FMB) with a second person (AET, GMR or VM) invigilating the test subject in the arena and managing the air and water temperatures in the room and aquaria.

3.3.4. Ethics and permits

The study was performed under the regulations approved by the Committee of Animal Experimentation of the University of Porto (Portugal) under the Directive 2010/63/EU of the European Parliament. Animal sampling was carried out under the permit numbers 27497/2019/DRNCN/DGEFF and SGIB/AF from *Instituto da Conservação da Natureza e das Florestas* (Portugal) and *Consejería de Agricultura, Ganadería y Pesca y Desarrollo Sostenible de la Junta de Andalucía* (Spain).

The described protocol was specifically devised to minimise invasiveness. All processes involving the animals were less invasive than the insertion of a needle. Furthermore, at the end of the study, all the geckos were unharmed and released back to their respective sites of capture.

3.3.5. Data retrieval and statistical analyses

IR videos were analysed *a posteriori* with FLIR Tools software (version 5.13.18031.2002). Using the *Spotmeter* tool, temperature data was extracted from one frame every 20 seconds for seven different body parts: snout (tip), right eye, head (centrally, above parietal scales), mid-dorsum, right leg (knee), right foot (centrally) and tail (dorsally, above the cloaca) (Fig. 3.3). For this analysis, skin emissivity was set at 0.96, following previous works from the authors (Barroso et al., 2020), as well as a distance of the camera to the subject set to 30 cm and ambient temperature left at the software's pre-set 18ºC. All cooling videos were processed by VM and all heating videos processed by AET, since these two heat exchange processes were analysed separately. For consistency, prior to the video analysis all authors agreed on the reference points used to define the exact locations from which temperatures would be extracted in

different body parts. Additionally, and to standardise the thermal video processing protocol, VM and AET analysed a practice sub-set of the videos together, prior to the final video analysis. A detailed description of a since enhanced version of the thermal video processing protocol is available in the online protocol repository https://dx.doi.org/10.17504/protocols.io.n2bvj358xlk5/v1 (Barroso, 2024).

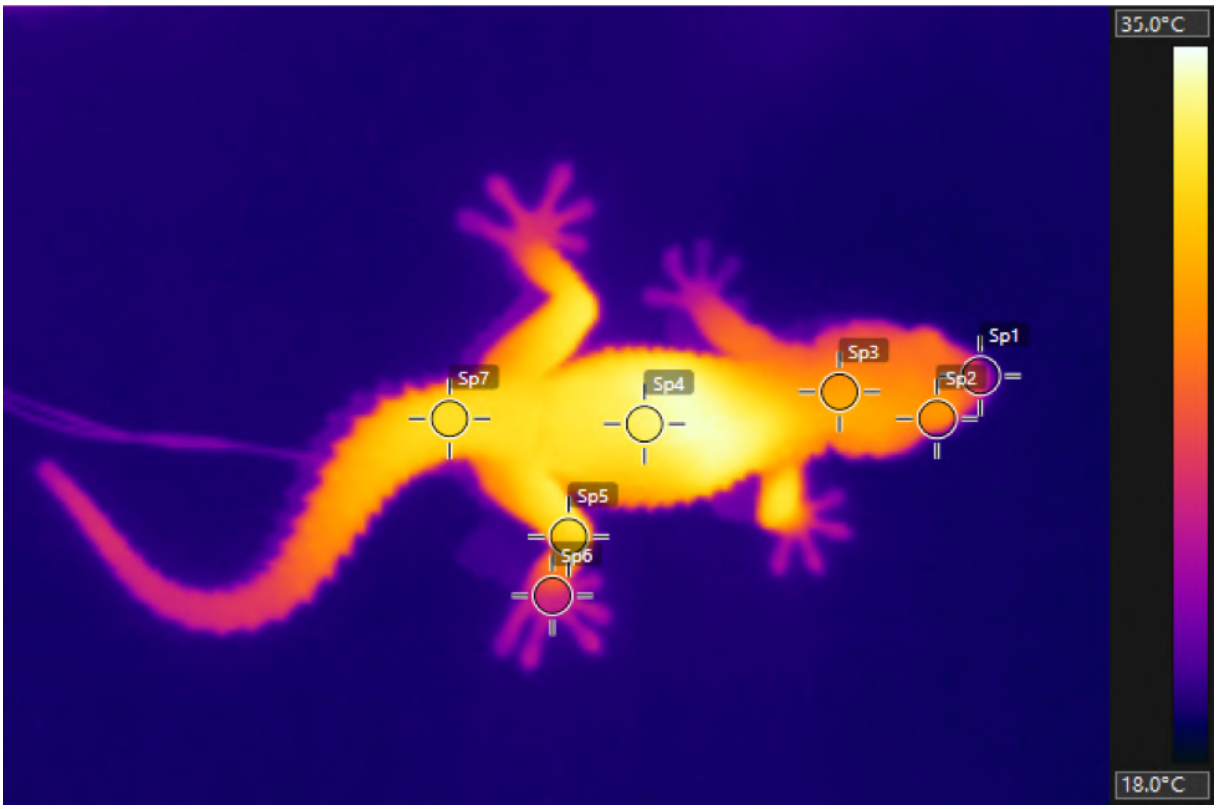

Fig. 3.3: Thermal image of *T. mauritanica* obtained from a frame of one of the thermal videos recorded. Radiometric image showing the placement of the measurement points for each body part: Sp1 - snout, Sp2 - right eye, Sp3 - head, Sp4 - mid-dorsum, Sp5 - right leg, Sp6 - right foot and Sp7 - tail.

Before any statistical analysis was conducted, raw absolute temperatures were converted into cumulative temperature changes so that starting temperature change is zero (i.e. *Cumulative Temperature at time*$_x$ = *Temperature at time*$_x$ - *Temperature at time*$_0$), thus correcting for potential differences in the starting temperature of the different animals and between different body parts. Heating and cooling trials were treated as separate datasets as these refer to different processes.

Paired T-tests between initial and final mass (per treatment) were carried out to explore if animals experienced significant mass losses during the trials as large changes in mass might also represent changes in the animal's thermal inertia.

Linear Mixed-Effect models were applied to each dataset independently, with cumulative temperature change as the dependent variable, using the R package *lme4* v1.1-35.1 (Bates et al., 2015). Original full models contained all the measured variables: time, treatment (heliothermic vs thigmothermic), body part, defecation, posture, initial mass and population. Additionally, relevant interactions between variables were considered, and individual ID added as a random factor to account for the repeated measures. Time was coerced to follow a polynomial function (second-order orthogonal polynomial), since it considerably increased the fit of the model, as expected from the literature (Bakken & Gates,1974). A backward stepwise regression approach (Draper & Smith, 2015) was used for model selection whereby nested models were compared based on a chi-squared test between the log-likelihood of the two models. This sequential process was undertaken until a minimal model was reached (independently for each heat exchange process). Once the final model was selected, residuals were verified to accomplish the assumptions of homoscedasticity and normality, and the p-values for the minimal models' explanatory variables computed with the R package *lmerTest* v3.1.3 (Kuznetsova, Brockhoff, & Christensen, 2017). The effect size of each such variable was calculated with the R package *effectsize* v0.8.6 (Ben-Shachar, Lüdecke, & Makowski, 2020).

A second analysis was carried out with a data subset containing only one minute of trial, namely, the time interval 60-120s, since the highest rates of heat exchange are expected when the difference in temperature between the animal and its environment is the largest (i.e. the start of the trial). However, the first minute was discarded since the animals were often still getting used to the set-up and therefore moved considerably, resulting in a higher amount of time performing exploratory, non-thermoregulatory behaviours.

Ultimately, this allowed us to investigate the rapid (maximal) rates of temperature change in each body part by comparing the gradient of temperature change per time for each body part. The same statistical pipeline, described for the full dataset, was applied to the maximum rates dataset. An additional step included the calculation of the Ordinary Least Squares (OLS) regression slopes for the relationship between temperature change and time (i.e. the rate of temperature change) for each body part, under each treatment (heliothermy vs thigmothermy) for each heat exchange process (heating vs cooling). The slopes, intercepts and corresponding 95% confidence intervals (CI's) were calculated using the R package *lmodel2* v1.7-3 (Legendre, 2018).

Finally, graphs were plotted using the *ggplot2* v3.4.4 package (Wickham, 2016). All analyses for this study were conducted in R software, version 4.3.2 (R Core Team,

2023). All data and R scripts for statistical analyses are openly available in the following GitHub repository: https://github.com/FredericoMB/Tarentola_Thermal_Exchange_Rates. Thermal videos available upon request.

3.4. Results

Paired T-test comparisons between initial and final mass, for each treatment, did not show any significant differences (Heliothermy: t = -0.061, p-value = 0.95, df = 78; Thigmothermy: t = -0.081, p-value = 0.93, df = 78). Hence only initial mass (i.e. henceforth “mass”) was considered for the remaining analyses.

3.4.1. Full dataset

Different heating and cooling patterns were observed among body parts (Fig. 3.4). During heliothermic heating, the cumulative increment of temperature achieved a plateau around 200-300 seconds after starting the experiment (Fig. 3.4).

However, the magnitude of total temperature change differed between body parts. In general, the foot showed half the temperature increase when compared to the heating of the other body parts, while the snout showed an intermediate position. For the cooling trials, a similar but inverted pattern was observed (Fig. 3.5).

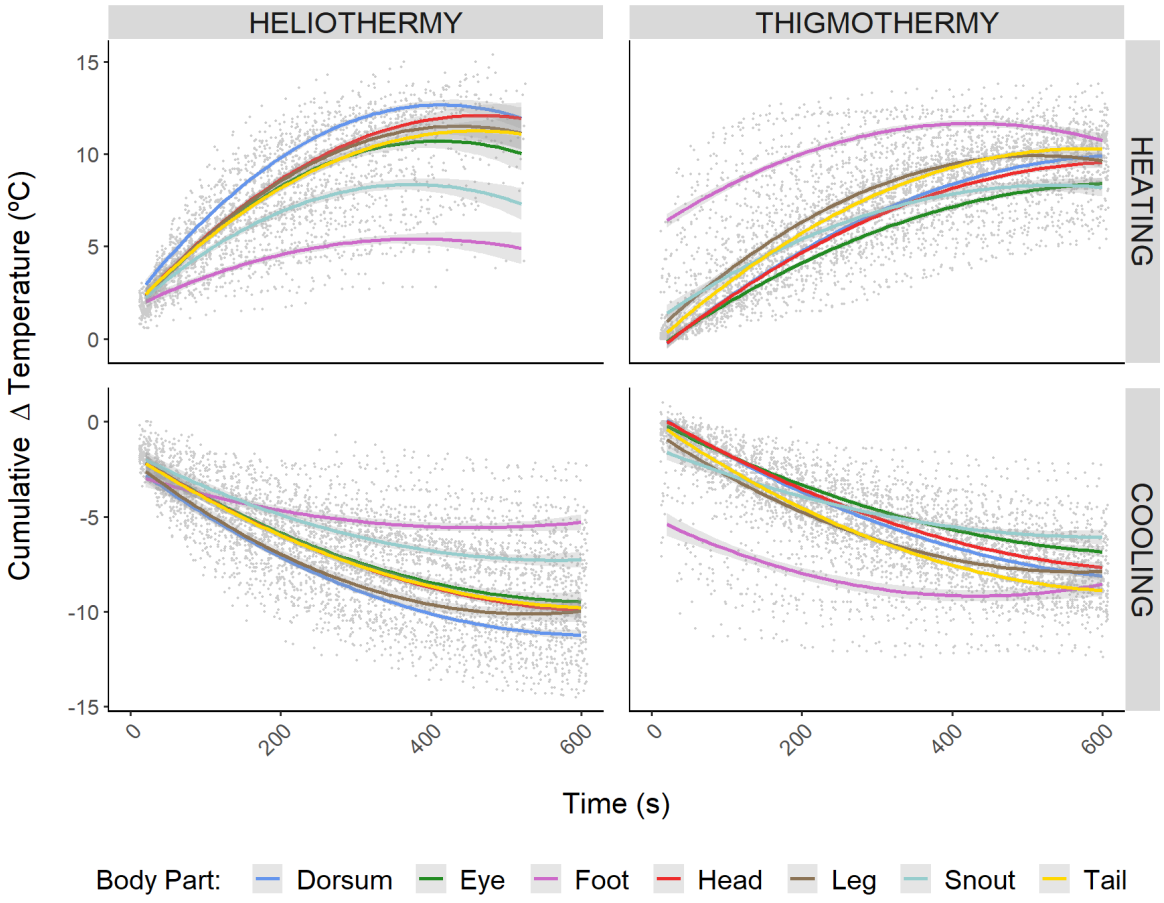

Fig. 3.*4*: Plots showing the cumulative temperature change over time. The figure shows the cumulative temperature change (i.e. Cumulative Temperature at $time_x$ = Temperature at $time_x$ - Temperature at $time_0$) in both heliothermic (left column) and thigmothermic (right column) experiments, during heating (top row) and cooling (bottom row) processes. Lines represent a second order polynomial regression between the cumulative temperature change against time, for each of the measured body parts (given by the different colours) and with the shaded area around the lines representing the corresponding standard error. Points represent cumulative temperature change of each body part from each individual, shown here purely for a graphical representation of the data's dispersion.

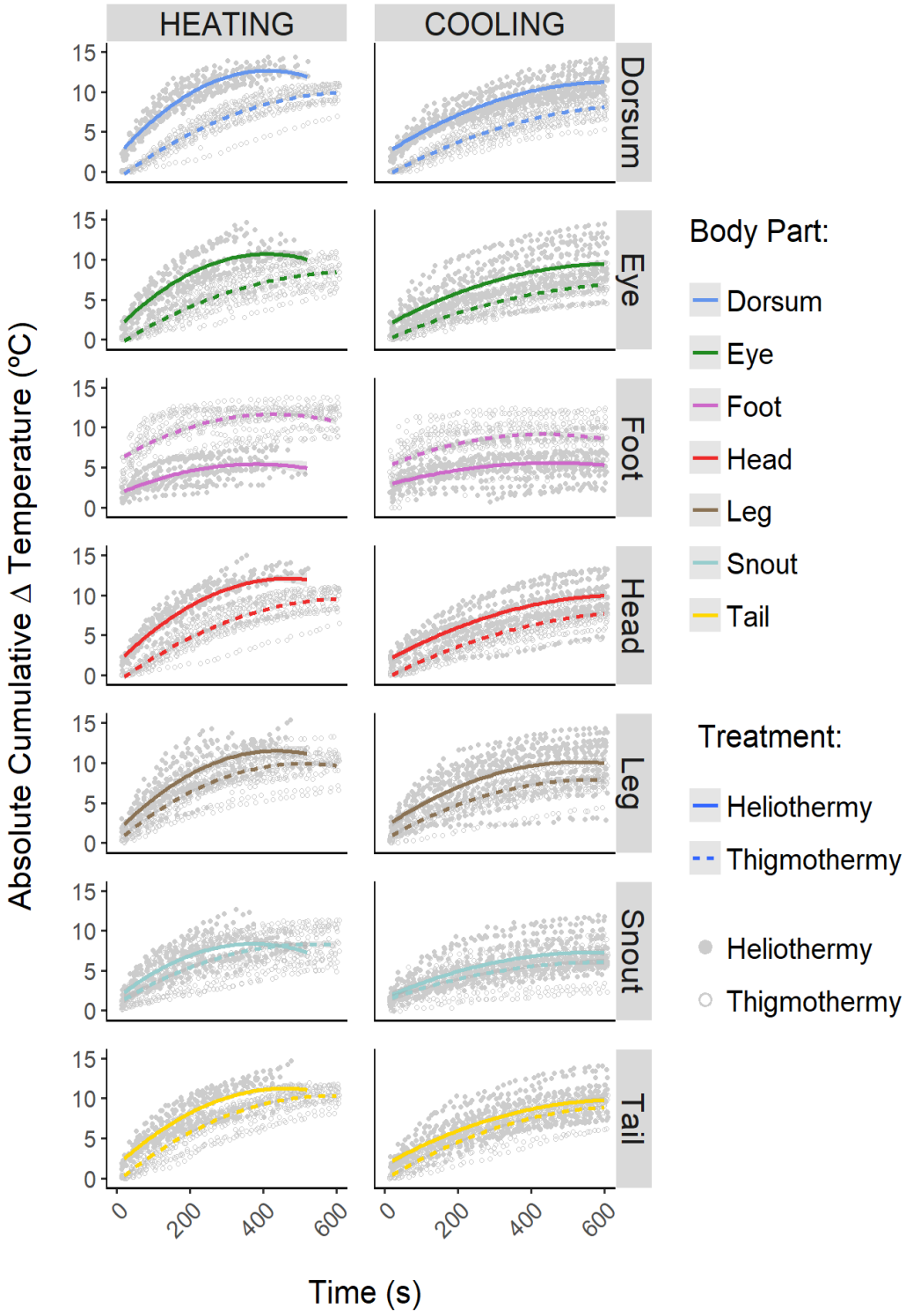

Fig. 3.5: Thermal exchange rate graphs per body part. Heating (left column) and cooling (right column) profiles for the different body parts, under heliothermic (full line, full points) and thigmothermic (dashed line, empty points) treatments. 2nd order polynomial fitted to demonstrate the trend in the data.

Overall, the foot was the body part showing the least temperature decrease, while the temperature of the snout decreased 1.5 times more than that of the foot and all the remaining body parts decreased double the amount than that of the foot. In general, the heating of the heliothermic experiment tended to finish earlier than the cooling since, for heliothermic heating, individuals often achieved the 35ºC threshold before reaching the 10th minute of the trial.

Contrastingly, during the thigmothermic heating experiment, the foot attained higher temperatures and much sooner than other body parts. For the foot, within the first 200 seconds, the cumulative increment of temperature was at least double than that of the second warmest body part (Fig. 3.4). However, it is worth noting that the y-intercept of the 2nd order polynomial fitted to the foot thigmothermic heating data was considerably different from zero, thus hinting at a very rapid increase in the foot temperature within the first 20 seconds of the trial. The cooling part, however, showed the inverted pattern, as previously observed during the cooling of the heliothermic experiment. Interestingly, during heating, the mean increment of cumulative temperature among all body parts for both heliothermic and thigmothermic experiments was very similar (10.27 ± 2.61ºC vs 9.91 ± 1.72ºC respectively). Conversely, during cooling, animals in the heliothermic experiment lost almost 1.5ºC more than during the thigmothermic experiment.

Model selection for the full dataset yielded similar results between the two heat exchange processes (heating vs cooling). Both models kept *individual ID* as a significant random factor. The heating model kept the independent variables of *time* [1st and 2nd order polynomial terms, hereafter *poly(time, 2)*], *treatment* (heliothermy vs thigmothermy), *body part*, *defecation* and *mass,* as well as the interactions between *poly(time, 2)* and *treatment*, *poly(time, 2)* and *body part*, *treatment* and *body part*, and the triple interaction between *poly(time, 2)*, *treatment* and *body part*. All such variables and interactions, with the exception of *mass*, were statistically significant (Table 3.1). The cooling minimal model included the same variables and interaction terms as the heating minimal model previously described, with the addition of the *posture* variable (Table 3.1). An ANOVA of the variables in this model, showed that again, the *mass*, but also the *poly(time, 2)* x *treatment* interaction, were not significant (Table 3.1).

		HEATING			COOLING		
Dataset	**Variable(s)**	**Deg. Freed.**	**F-value**	**Pr(>F)**	**Deg. Freed.**	**F-value**	**Pr(>F)**
Full Dataset	poly(Time, 2)	2, 6375.0	577.9081	**<2.2e^{-16}**	2, 7624.6	506.9119	**<2.2e^{-16}**
	Treatment	1, 6387.8	672.9938	**<2.2e^{-16}**	1, 7639.5	1686.6309	**<2.2e^{-16}**
	Body Part	6, 6373.7	97.3144	**<2.2e^{-16}**	6, 7624.7	186.6488	**<2.2e^{-16}**
	Mass	1, 28.5	0.4389	0.5130	1, 29.8	0.0971	0.7575
	Defecation	1, 3454.8	14.6978	**0.0001**	1, 4483.3	76.0981	**<2.2e^{-16}**
	Posture	*NA*	*NA*	*NA*	1, 7327.9	28.0999	**1.186e^{-7}**
	poly(Time, 2) x Treatment	2, 6378.3	87.3184	**<2.2e^{-16}**	2, 7625.0	1.5557	0.2111
	poly(Time, 2) x Body Part	12, 6373.3	67.6620	**<2.2e^{-16}**	12, 7624.3	86.9433	**<2.2e^{-16}**
	poly(Time, 2) x Mass	2, 6374.7	4.2508	**0.0143**	2, 7624.7	12.8476	**2.690e^{-6}**
	Treatment x Body Part	6, 6373.7	633.9881	**<2.2e^{-16}**	6, 7624.9	577.4286	**<2.2e^{-16}**
	poly(Time, 2) x Treatment x Body Part	12, 6373.3	7.7794	**1.447e^{-14}**	12, 7624.3	3.5849	**2.324e^{-5}**
Max. Rates Dataset	poly(Time, 1)	1, 759.87	681.482	**<2.2e^{-16}**	1, 630.08	476.3650	**<2.2e^{-16}**
	Treatment	1, 761.42	16.837	**4.512e^{-5}**	1, 641.54	22.0614	**3.232e^{-9}**
	Body Part	6, 758.16	14.249	**2.002e^{-15}**	6, 627.89	8.5732	**5.662e^{-6}**
	Population	*NA*	*NA*	*NA*	3, 15.50	2.8506	0.0714
	Treatment x Body Part	6, 757.83	21.014	**<2.2e^{-16}**	6, 627.85	4.6467	**0.0001**

Table 3.*1*: ANOVA tables for the independent, fixed effects variables in the minimal models for each heat exchange process (heating vs cooling) in each of the datasets (full vs maximum rates).

Bold p-values indicate significance at alpha < 0.05. Note that the variable poly(Time, 2) in the full dataset minimal models, differs from the poly(Time, 1) in the minimal models for the maximum rates dataset. The former refers to two orthogonal polynomials (1st order and 2nd order) fitted , and hence uses 2 degrees of freedom (one for each curve) , while the latter only fits one [1st order] polynomial only, thus only using 1 degree of freedom, as the 2nd order polynomial was deemed non-significant (Heating: χ^2 = 0.0508, p = 0.8216; Cooling: χ^2 = 0.0159, p = 0.8996) during the model selection process. Degrees of freedom estimated with Satterthwaite's method (Satterthwaite, 1946) and reported as "Numerator d.f., Denominator d.f.".

Further exploring the magnitude of the effect size of each variable, in each minimal model, demonstrated that the *poly(time, 2)* terms had consistently large effects for both heating and cooling models (Fig. 3.6). Additionally, the interactions of *poly(time, 2)* with the *body part* variable, as well as with the *body part* x *treatment* variables, all showed large effect sizes (Fig. 3.6). Nonetheless, these variables, and especially the *time* polynomials, showed particularly broad 95% CI's (Fig. 3.6), displaying large variability of the effect size of these parameters.

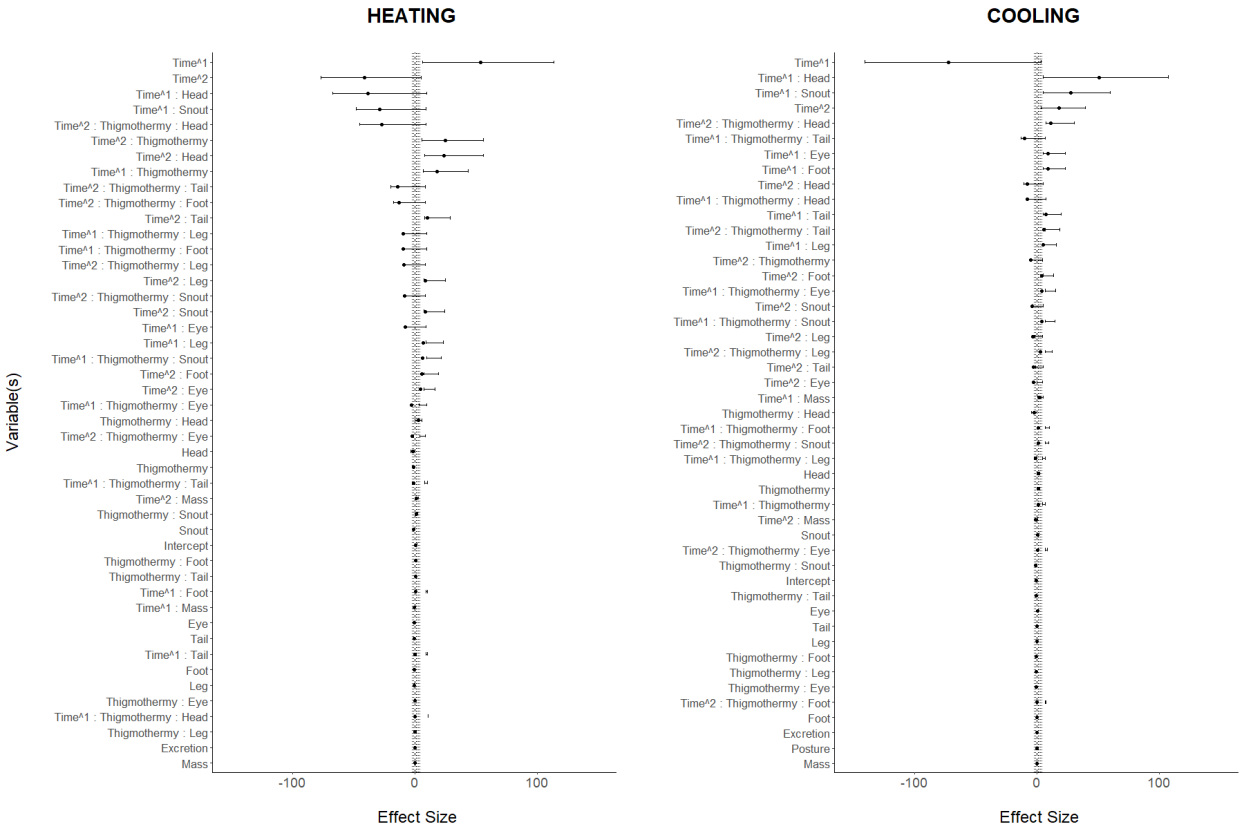

Fig. 3.6: *Effect size plot for the variables of the minimal linear mixed effects model for the full heating and cooling rates datasets.*

3.4.2. Maximum rates dataset

For maximum rates dataset, overall absolute cumulative temperature changes in the thigmothermic trials were both slower and less divergent among body parts, when compared to those observed for the heliothermic trials (Fig. 3.7). Additionally, within each heating type, thermal exchange rates of different body parts were also more similar within the cooling trial than within the heating trial (Fig. 3.5). Again, the foot showed the most divergent results among the different body parts (Fig. 3.7) with a higher heating rate for the thigmothermic process than for the heliothermic one.

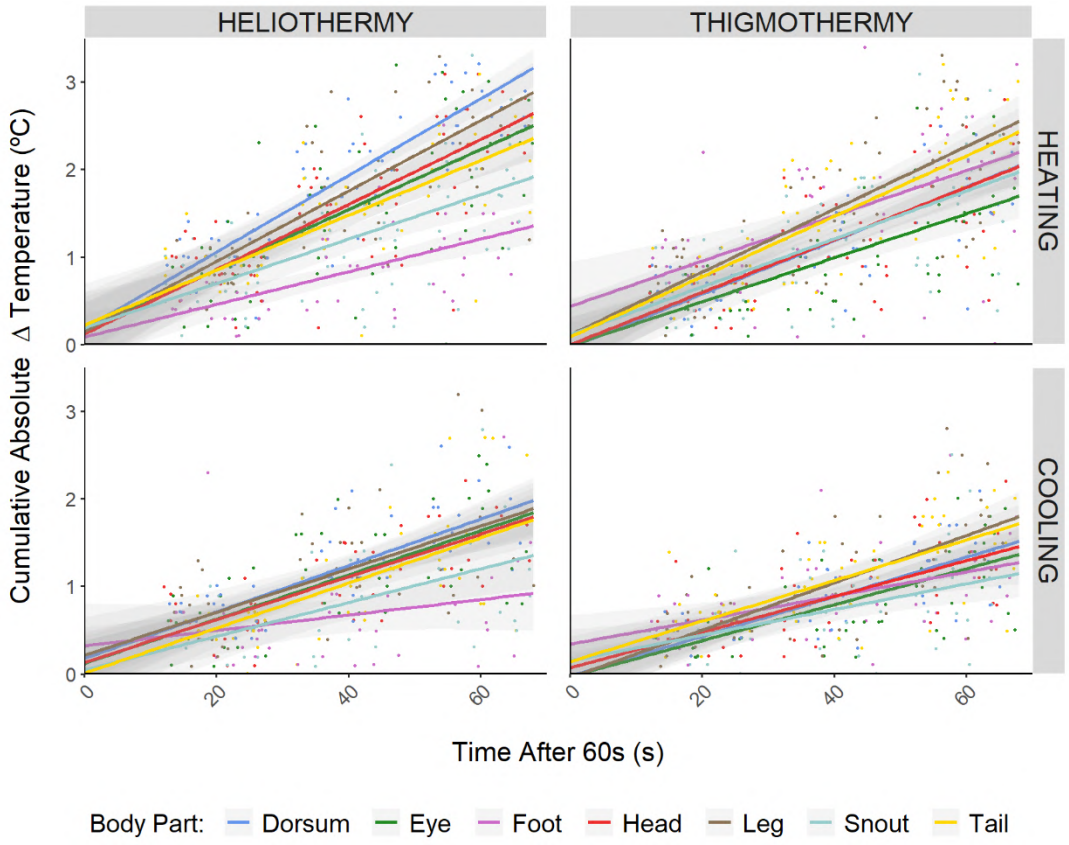

Fig. 3.7: *Absolute cumulative temperature change per time for maximum rates dataset. These plots show the absolute cumulative temperature change for one minute, after the first 60 seconds of the experiment (i.e. | Temperature at time$_x$ - Temperature at time$_{60}$ | ; where 60s ≤ x ≤ 120s), representing the maximum rate of heat exchange of each body part, in both heliothermic (left column) and thigmothermic (right column) experiments during heating (top row) and cooling (bottom row) processes. Lines represent the linear regression between the cumulative absolute temperature change against time, for each of the measured body parts (given by the different colours) and with the shaded area around the lines representing the corresponding standard error. Points represent the cumulative absolute temperature change for each body part of each individual.*

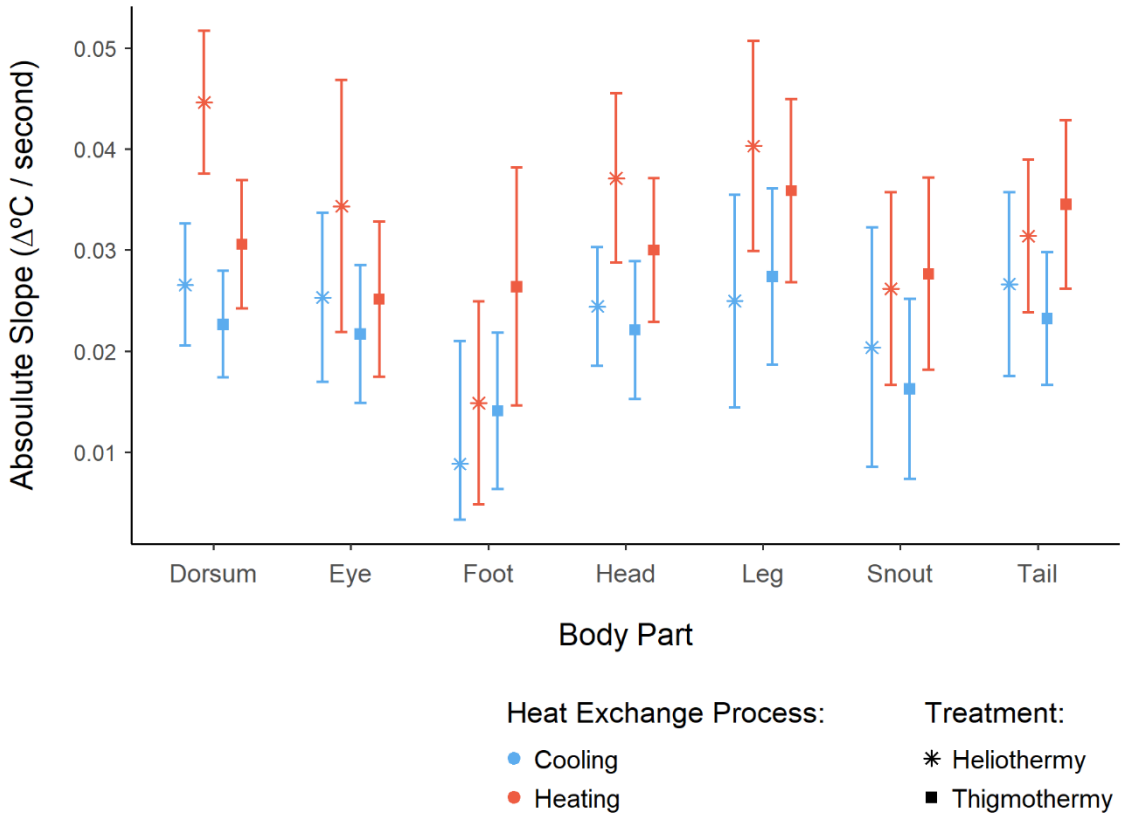

Fig. 3.8: *Slopes and corresponding 95% confidence intervals, for the maximum heating (red) and cooling (blue) rates, under the heliothermic (star) or thigmothermic (square) treatments. These slopes were obtained from the Ordinary Least Square (OLS) regression models. Absolute values were used to facilitate direct comparison of the slope's magnitude, since the cooling and heating regressions would have opposite trends given the directly contrary directionality of temperature change over time for these two processes.*

Furthermore, the foot's heliothermic heating rate, as given by the absolute slope of the respective OLS regression (Fig. 3.9), was the lowest heating rate of any body part in any of the treatments (Fig. 3.8). Conversely, the dorsum under heliothermic heating exhibited the fastest thermal exchange rate of any body part, under any treatment (Fig. 3.7 and Fig. 3.9). Additionally, the dorsum also showed the most marked difference between the heating rates of the two treatments (Fig. 3.8).

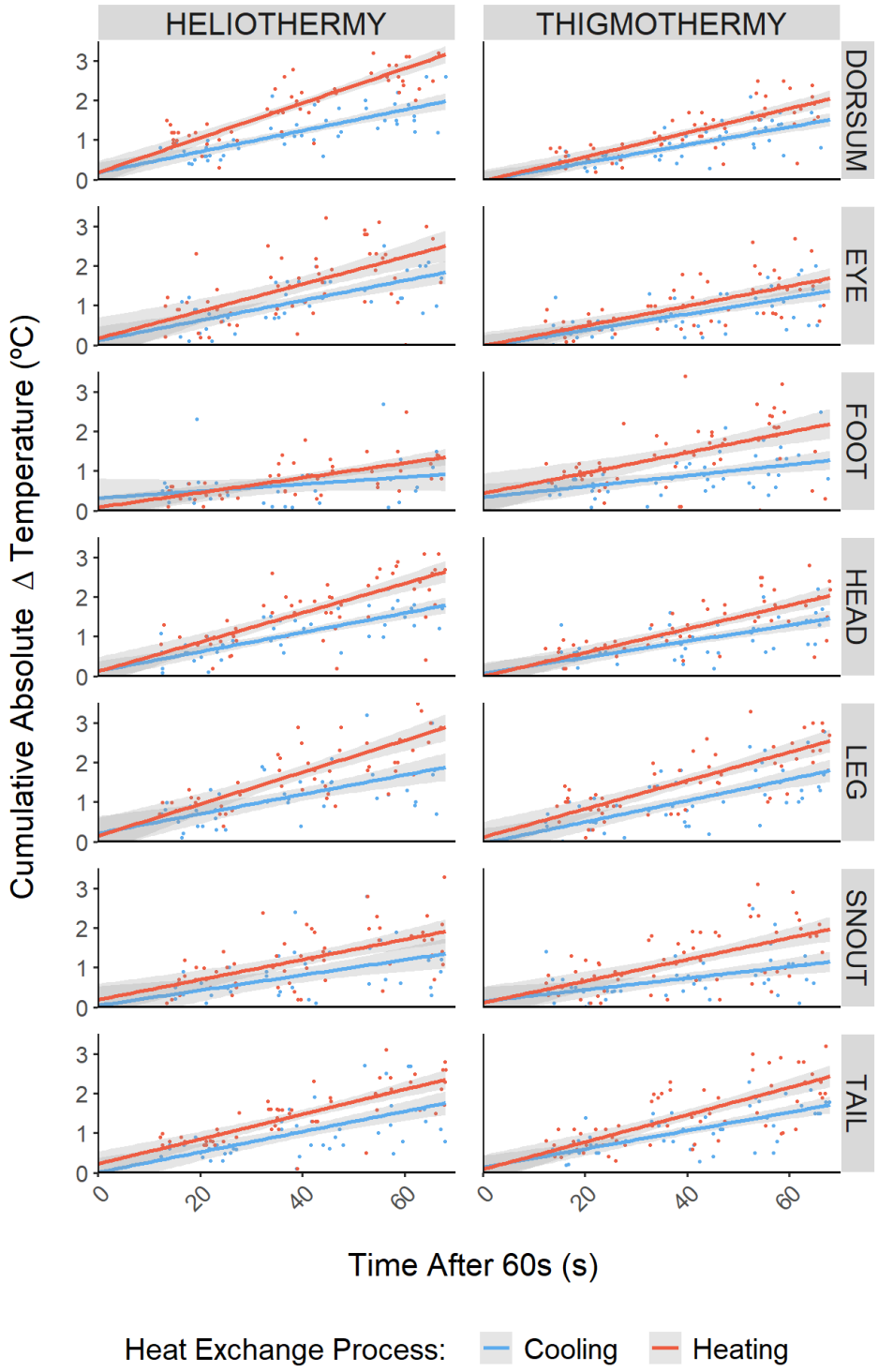

Fig. 3.9: *Graphical representation of the OLS regression lines (and 95% CI's in grey shade) for the maximum rates of cooling (blue) and heating (red). Absolute rates are plotted in order to facilitate the comparison between the magnitude of the slope between the heating and cooling processes.*

The OLS regression showed no intercept deviating significantly from zero (Table 3.2), but slope values were more variable. The strength of the linear relationship varied extensively (0.06 ≤ R2 of OLS regressions ≤ 0.74) depending on the body part, treatment and heat exchange process (Table 3.2) and was generally weak (overall mean R2 ± SE = 0.44 ± 0.03). Heating showed stronger relationships than cooling (mean R^2 ± SE for heating = 0.48 ± 0.04 vs for cooling = 0.41 ± 0.04). The foot consistently had a very weak fit, showing the lowest R^2 values (mean R^2 ± SE = 0.18 ± 0.04) of all other body parts (Table 3.2), as opposed to the dorsum, which showed the best fit in all regressions (Table 3.2), with its lowest fit for thigmothermic cooling (R^2 = 0.59) and its highest fit for heliothermic heating (R^2 = 0.74) demonstrating a good overall fit on all its models (mean R^2 ± SE = 0.65 ± 0.03).

		HEATING					COOLING				
Treatment	**Body Part**	**Slope**	**Slope 95% CIs**	**Intercept**	**Intercept 95% CIs**	**R^2**	**Slope**	**Slope 95% CIs**	**Intercept**	**Intercept 95% CIs**	**R^2**
Heliothermy Experiment	Eye	0.034	0.022 0.047	0.159	-0.379 0.697	0.35	-0.025	-0.034 -0.017	-0.119	-0.472 0.234	0.46
	Snout	0.026	0.017 0.036	0.147	-0.265 0.560	0.35	-0.020	-0.032 -0.009	0.020	-0.482 0.521	0.25
	Head	0.037	0.029 0.045	0.118	-0.243 0.478	0.59	-0.024	-0.030 -0.019	-0.131	-0.376 0.114	0.61
	Dorsum	0.045	0.038 0.052	0.138	-0.167 0.444	0.74	-0.027	-0.033 -0.021	-0.176	-0.428 0.075	0.62
	Leg	0.040	0.030 0.051	0.144	-0.305 0.593	0.55	-0.025	-0.035 -0.014	-0.195	-0.630 0.240	0.37
	Foot	0.015	0.005 0.025	0.135	-0.299 0.569	0.15	-0.009	-0.021 0.003	-0.320	-0.805 0.166	0.06
	Tail	0.031	0.024 0.039	0.219	-0.107 0.545	0.57	-0.027	-0.036 -0.018	0.038	-0.345 0.422	0.43
Thigmothermy Experiment	Eye	0.025	0.017 0.033	-0.014	-0.346 0.318	0.44	-0.022	-0.028 -0.015	0.091	-0.204 0.386	0.44
	Snout	0.028	0.018 0.037	0.098	-0.313 0.510	0.37	-0.016	-0.025 -0.007	-0.052	-0.438 0.334	0.23
	Head	0.030	0.023 0.037	-0.004	-0.312 0.305	0.57	-0.022	-0.029 -0.015	0.021	-0.274 0.316	0.45
	Dorsum	0.031	0.024 0.037	-0.034	-0.308 0.240	0.65	-0.023	-0.028 -0.017	0.024	-0.204 0.253	0.59
	Leg	0.036	0.027 0.045	0.111	-0.281 0.503	0.52	-0.027	-0.036 -0.019	0.058	-0.319 0.435	0.45
	Foot	0.026	0.015 0.038	0.409	-0.102 0.919	0.26	-0.014	-0.022 -0.006	-0.323	-0.658 0.013	0.23
	Tail	0.035	0.026 0.043	0.086	-0.276 0.448	0.54	-0.023	-0.030 -0.017	-0.138	-0.424 0.147	0.49

Table 3.2: *Model output table of the Ordinary Least Squares (OLS) regressions for the maximum thermal exchange rates dataset. The slope of the OLS regressions represents the rate of heat transfer.*

Model selection on the linear mixed effects models for the heating and cooling maximum rates datasets produced simpler minimal models than those attained for the full dataset (Table 3.1). Again, both minimal models kept *individual ID* as a significant random factor, and both models differed only by one variable - in this case, *population,* which was absent from the maximum heating rates model but present, albeit ultimately non-significant, in the maximum cooling rates minimal model (Table 3.1). Much like the full dataset minimal models described previously, *time* proved to be a highly significant variable (*poly(time, 1)*) for the maximum rates models as well. However, in this case, the 2nd order polynomial was deemed non-significant during the model selection process (Heating: χ^2 = 0.0508, p = 0.8216; Cooling: χ^2 = 0.0159, p = 0.8996). The remaining significant variables, present on both heating and cooling maximum rates' minimal models, were *treatment*, *body part* and their interaction (Table 3.1).

Again *time* showed the largest effect size by an entire order of magnitude, while all other variables had much smaller and similar effect sizes. Furthermore, in addition to showing the largest effect size, *time* once more showed the largest range in the magnitude of its effect.

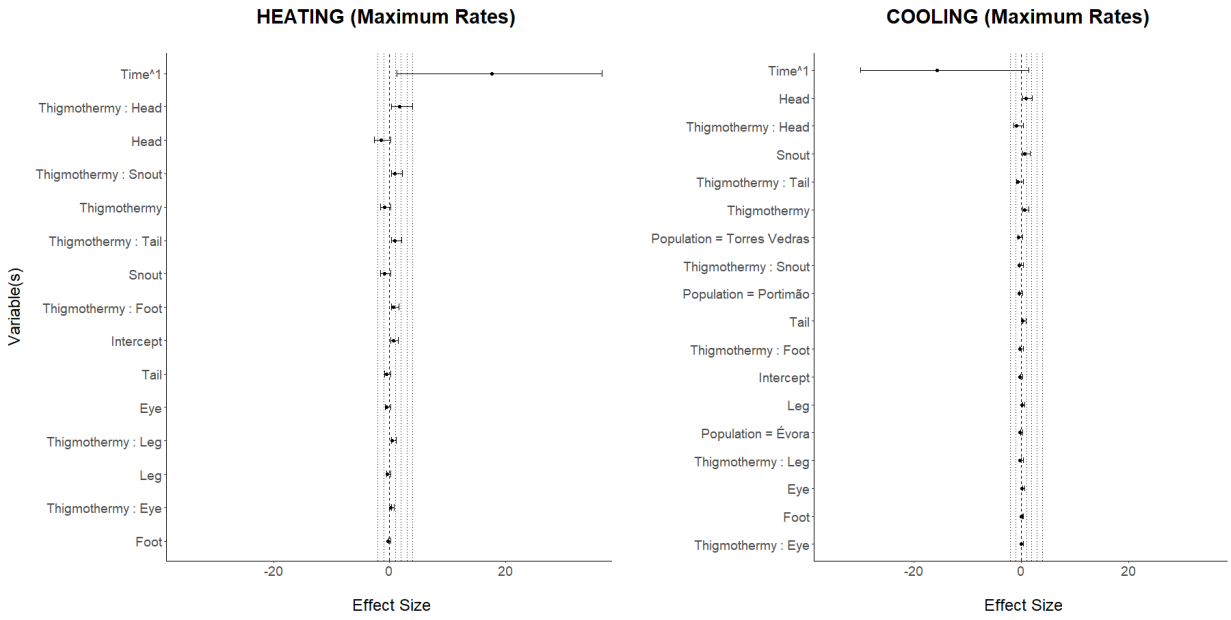

Fig. 3.10: *Effect size plot for the variables of the minimal linear mixed effects model for the heating and cooling maximum rates datasets.*

3.5. Discussion

Despite the generally acknowledged vital role that thermal exchange rates play in ectotherm ecophysiology (Bakken & Gates, 1975; Porter & Gates, 1969), traditional methods for estimating these often fail to provide realistic insights into the integrative interplay between the environmental, behavioural and physiological variables which modulate them. This was likely due to the technological limitations when most such classic thermal exchange rate studies were developed, particularly when applied to small ectotherms. However, with the onset of new tools such as thermal imaging (Tattersall, 2016), such evidence may be revised in the light of fine-tuned thermal data, at both spatial and temporal scales, in order to provide a more in-depth and integrative perspective into these and other thermophysiological phenomena (Tattersall & Cadena, 2013).

Here a replicable methodology to study thermal physiology in small to medium sized ectotherms is provided. This protocol allows examining thigmothermy and heliothermy separately, while under the same (i.e. comparable) methodological framework. Hence, this will enable disentangling the effect of thermal exchange strategies and of their underlying ecophysiological and behavioural variables. Ultimately, it will allow presenting a finer view into the thermal ecology of the studied animals.

Another major contribution is the ability to quantify thigmothermic heat exchange, a thermal strategy mostly neglected in the literature due to the difficulties in incorporating it into classical studies (but see Belliure & Carrascal, 2002). This study opens an avenue to examine hundreds of species with thigmothermic behaviours, as well as to test if species previously thought to be strict heliotherms could also complement their preferred thermoregulatory strategy with thigmothermic thermal exchanges.

Specifically for the model species, our results reveal different thermal patterns depending on the body part (Fig. 3.4 and Fig. 3.8), providing further evidence for regional heterothermy (Heath, 1964) in small lizards, in line with what has been reported in other squamates (e.g. Cox et al., 2023; Sannolo et al., 2014). Of the body parts studied, the foot experienced the slowest and smallest cumulative change in temperature in the heliothermic heating and cooling processes (Fig. 3.4). This was expected since, under heliothermy, the foot acts as a location of heat loss to prevent overheating. Being in direct contact with the cold substrate and given its small size, it is likely that the rate at which it is receiving heat from the rest of the body may be very similar to the rate at which it is losing heat to the cold substrate, thus buffering the range of temperatures experienced.

A similar pattern is also observed for the thigmothermic cooling of the foot. However, having the slowest rate of the body parts monitored, an intercept considerably different from zero, and a nearly straight-line pattern (i.e. the least curved of all the body parts' regression lines), hints at its high sensitivity to temperature change. Additionally, the foot was the body part showing the fastest heating rate during the thigmothermic heating trials, as expected due to its small size and concordant with previous comments on the sensitivity to temperature. Its small size, and hence small inertia, suggest that it is less likely to accumulate "thermal history" (i.e. be less affected by past states and conditions) than other body parts and quickly equilibrate with its environment (Bartholomew & Tucker, 1964; Bell, 1980; Grigg, Drane, & Courtice, 1979).

The differences between the foot's cooling processes (post-heliothermic vs post-thigmothermic heating) are also worth noting. They could be purely biophysical - i.e. the foot had different thermal starting points simply because the resulting pattern of regional heterothermy differed between the two heating treatments - but may result from the gecko's ability to identify the main heat source and adjust its blood circulation accordingly in order to optimise the heat distribution along the body (Dzialowski & O'Connor, 1999; Dzialowski & O'Connor, 2004). If this were true, it would mean that the animals' physiology may be more "focused" on maximising heat gain rather than on minimising heat loss, as often reported in other reptiles (Dzialowski & O'Connor, 2001; reviewed in Seebacher & Franklin, 2005). Interestingly though, the leg follows the pattern observed in the remaining body parts. This may suggest vasoconstriction in the wrists as well as the presence of specific vascularisation patterns of the limbs (as already reported for other geckos in Russel, 1981). The latter may be linked to the maintenance of proper blood irrigation and hence of the adhesive function of the lamellae in geckos (Autumn & Peattie, 2002). Alternatively, it may function as counter-current heat exchange systems, as found in other animals (Holowatz & Kenney, 2010; Schmidt-Nielsen, 1981; Tattersall, Cadena, & Skinner, 2006), allowing for a fine control of thermal exchange at this site.

Regarding the thermal exchange rates at other body parts, during the heliothermic heating experiment, the dorsum showed the fastest heating rate as well as the widest thermal range (Fig. 3.4 and Fig. 3.8). This was expected as reptiles utilise their dorsum as a main area for radiant heat absorption. Hence, they may capitalise on the dorsum's large thermal inertia to utilise it as a thermal reservoir from which heat can be redistributed to other parts of the body. Furthermore, the dorsum was the body part that experienced the fastest and largest temperature shift during the post-heliothermic cooling trial (Fig 3.5), further supporting the thermal reservoir hypothesis since it can

accumulate excess heat when basking, and then redistribute it once the external heat source was no longer available.

As for the thigmothermic heating and cooling, the dorsum followed an intermediate/ average rate and range of temperature, which is in line with its large inertia and the fact that it is not directly an area of heat acquisition. The eye - shown to be a good proxy of internal body temperature for this (Barroso et al., 2020) and other (Barroso et al., 2016) lizards - and the snout also show comparatively lower thermal ranges and slower heat exchange rates than other body parts (except the foot). This is compatible with the presence of surface-to-core temperature gradients, but might also result from evaporative heat loss (Tattersall, Cadena, & Skinner, 2006), which would allow for faster cooling of these areas, even while the whole animal is heating up. The latter has been hypothesised as a strategy to avoid the overheating of sensitive, vital organs such as the brain (Clarac & Quilhac, 2018; Sannolo et al., 2014; Tattersall, Cadena, & Skinner, 2006). If, however, evaporation from ocular and nasal surfaces led to significantly increased rates of heat loss in these structures, then this should also be reflected in the cooling trials as faster cooling rates of these body parts. In fact, the opposite was observed. This further reinforces the idea that inner body temperature tends to be more stable and takes longer to respond to changes in the external thermal environment, even in a smaller-bodied animal, but could also result from minimal ocular evaporation due to the presence of a scale (the brille) covering the eyes – a common feature among geckos (Bellairs, 1948).

In general, for all body parts but the foot, heliothermic processes (both heating and cooling) were faster and showed wider thermal ranges (Fig. 3.4 and Fig. 3.8). Nonetheless, this might be artefactual since the two set-ups were not emitting the same power. Hence, the biological significance of this difference cannot be discussed here. Arguably, though, this may still represent an ecologically realistic scenario since, if a surface is “too hot for comfort”, the animal will likely not remain still as it may burn its feet (Vasconcelos, Santos, & Carretero, 2012). Contrastingly, even in very hot sun, a cold ectotherm could be found basking for the amount of time needed to obtain a temperature within its set-point range (Carrascal et al.,1992; Gans & Pough, 1980), as long as the substrate itself is not too hot. Nonetheless, future deployments of this experimental framework may consider adjusting the power and/or distance of the heat source so that the resultant operative temperature is the same between treatments. Furthermore, colouration should also be monitored, as it can influence the rate of radiant heat transfer (Gates, 1980; but see Hastings et al., 2023; Rutschmann et al., 2020). This may be of

particular relevance for species that exhibit rapid physiological colour change (Sherbrooke & Frost, 1989) whereby the animal can rapidly darken or lighten its skin according to its ecophysiological needs, as reported even for the species tested here (Fulgione et al., 2019; Vroonen et al., 2012).

3.6. Conclusion

Ultimately, with this study we present a new and replicable methodological procedure to infer heat exchange through heliothermy and thigmothermy in ectotherms. This novel method successfully integrated environmental, physiological and behavioural variables, within an experimental context, in order to precisely describe thermal exchange rates at a high spatial (across different body parts) and temporal resolution while relying on new technologies that minimise the invasiveness during data collection. Moreover, we provide a proof-of-concept of this methodology by testing it on a crepuscular and nocturnal ectotherm, where we were able to unravel the complexity and importance of regional heterothermy, showing that body parts within the body of a small lizard can behave differently depending on the heat source available, even in a short timeframe.

The ease of replicability of the described set-up, along with its flexibility, could enable future studies to adapt this method to investigate other factors (e.g. colouration, wind, body shape and condition, etc) potentially affecting thermal exchange rates and body heat distribution in ectotherms. The design may easily be extended to other organismal models, such as amphibians and non-flying arthropods, as well as simulate different environmental contexts (e.g. climate change, wind, presence of conspecifics, predation risk, etc). More importantly, this methodology is expected to further the knowledge of terrestrial ectotherm thermal physiology and test alternative hypotheses, representing a valuable tool for addressing thermal ecology questions - classical and new - with innovative, integrative and less stressful approaches.

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4. Chapter 4 - On the body temperatures of crocodilians: a quantitative evaluation of classical and novel methods, with a discussion of future perspectives

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4.1. Abstract

Thermoregulation in crocodilians is driven by a combination of behavioural, physiological, and anatomical mechanisms that enable these ectotherms to adapt to diverse environments. This study evaluated the use of classical and novel tools for measuring body temperature in crocodilians, resorting to *Crocodylus moreletii* as the studied species. The tools tested included cloacal probes, stomach loggers, subcutaneous sensors, external surface loggers, and infrared thermography (IRT). Data was collected from wild and captive individuals across a range of body sizes and the temperature of different internal and external body parts, measured through these different tools, compared through Ordinary Least Squares regressions. Linear mixed effects models were also employed in order to determine which variables affected the measured temperatures.

The results suggested that stomach loggers outperform cloacal measurements as a reference for internal temperature, having exhibited stronger correlations with subcutaneous and external temperatures. Axillary and neck temperatures emerged as the most reliable proxies for core temperature, with R^2 values of 0.790 and 0.890 respectively when regressed against stomach temperature. This could be due to their proximity to vascularized tissues and structures such as osteoderms. Conversely, IRT of the eye showed low correlations with internal [cloacal] temperature ($R^2 = 0.455$), likely due to regional heterothermy, cephalic vascular anatomy, and localised thermal inertia of the head, all of which may decouple ocular surface temperatures from the core's general thermal state.

Overall, these findings further confirm the presence of regional heterothermy in crocodilians, demonstrated by significant temperature gradients between body regions and their dynamic regulation via localized blood flow. Furthermore, this study emphasizes and discusses the need for species-specific methodologies in thermal ecology and underscores the potential of integrative biologging approaches to combine physiological, behavioural, and environmental data. Such approaches are essential for advancing the understanding of crocodilian ecology, particularly in the context of habitat changes and climate variability, and provide a foundation for improving conservation strategies.

4.2. Introduction

Crocodilians are remarkable examples of ectothermic adaptation, balancing physiological and behavioural thermoregulation strategies to maintain stable body temperatures across diverse and often extreme habitats (Grigg & Kirshner, 2015). As ectotherms, they rely primarily on external heat sources rather than internal metabolic processes for thermoregulation (Lang, 1987; Angilletta, 2010). This dependence on environmental temperatures necessitates a suite of behavioural and physiological mechanisms that, together with morphological features (see below), allow crocodilians to efficiently control heat exchange and its subsequent redistribution throughout the body. Much like in other reptiles, their ability to adapt to variable thermal conditions and maintain temperatures within an optimal range (Diefenbach, 1975), plays a crucial role in other physiological processes such as metabolic activity (Seebacher et al, 2003; Rodgers and Franklin, 2021), digestion (Lang, 1979), immune function (Lang, 1987), growth (Joanen & McNease, 1979; Angilletta et al, 2004), and reproduction (Lang, 1987; Seebacher, 1999; Grigg & Kirshner, 2015). Understanding the intricacies of crocodilian thermoregulation is, hence, essential, especially in the face of climate change, as climate shifts and habitat degradation could impact thermal availability (among many other factors), ultimately affecting the survival, distribution, and conservation status of extant crocodilian species (Payne et al. 2024).

Thermoregulatory behaviours such as basking, gaping, shuttling and selective posturing when in water, are fundamental to how crocodilians regulate their body temperature (Tb) (Spotila et al, 1977; Smith, 1979; Seebacher, 1999; Tattersall et al, 2006). By moving between sunlit and shaded areas, or between water and land, or even by adjusting their buoyancy to control the proportion of their body immersed and exposed to solar radiation (Smith, 1975, 1979; Seebacher 1999), crocodilians can modulate their temperatures based on their immediate thermal needs. These aid in maintaining relatively stable Tb's, usually within an optimal range - normally between ~28-34ºC for most species assessed (Johnson, 1974; discussed in Brien et al, 2012) - but see also the study by Campos and Magnusson (2013) where authors present evidence of an unusual thermal niche among extant crocodilians.

However, recent research is revealing that thermoregulation in crocodilians goes beyond mere behavioural adjustments. Studies have shown that crocodilians possess unique anatomical and physiological adaptations that facilitate efficient heat exchange and distribution. For example, the presence of highly vascularized osteoderms (bony plates

embedded within the skin), coupled with a responsive vasomotor control system which regulates blood flow to and from these structures, may play a crucial thermoregulatory role. By acting as sites of enhanced heat transfer, blood flow through these osteoderms can be modulated to either dissipate extra heat into cooler environments or retain it when basking (Farlow et al., 2010; Clarac & Quilhac, 2019).

Nonetheless, even without considering any physiological mechanism, the large adult body sizes attained by most extant species, result in an inherently high thermal inertia. This purely physical property, related to the size (i.e. mass) of the animal (Bell, 1980), means that large crocodilians are able to buffer short-term fluctuations in daily environmental temperatures, thus conferring to them a degree of homeothermy (Grigg et al, 1998). Hence, larger crocodilians are able to maintain temperatures above that of their environment without the behavioural costs associated with thermoregulation observed in smaller ectotherms, or of high resource consumption for constant endogenous thermogenesis, displayed by endotherms (Huey & Slatkin, 1976; but see Legendre et al, 2016, and Grigg et al, 2022 on the high metabolic rates and potential endothermy in ancestral archosaurs and amniotes, respectively). Indeed, thermal inertia due to size is also observed in other ectotherms such as giant tortoises (Don & Brown, 2017) and marine turtles (Paladino et al, 1990), and as has been hypothesized for large-bodied dinosaurs as well (Seebacher et al, 1999), being often referred to as gigantothermy (Paladino et al, 1990).

However, it is important to note that crocodilians hatch at a small size (~21-32cm total length, as reviewed by Somaweera et al, 2013; a size comparable to a medium-sized lizard). Yet, although juvenile and subadult growth rates are higher than adult growth rates (Web et al, 1978), they still depend on environmental conditions and resource availability (Grigg and Kirshner, 2015). As such, it may still take several years until an animal has reached a critical mass threshold (~20-30kg – Seebacher et al, 1999) above which thermal inertia may represent a significant contribution towards homeothermy. Moreover, even though most extant crocodilian species reach adult sizes well beyond such threshold (with *Paleosuchus* and *Osteolaemus* genera as notable exceptions), extinct species exhibited a much broader size range, with many species never reaching such threshold (Grigg & Kirshner, 2015). Furthermore, in addition to size diversity, extinct Crocodyliformes were also more diverse in habitats occupied (Mannion et al, 2019) with a well-represented fossil record for purely terrestrial (Grigg & Kirshner, 2015) and purely aquatic/marine species (Grigg & Kirshner, 2015; Séon et al, 2020), in addition to those with the amphibious lifestyle currently observed. These diverse lifestyle and habitat

preferences almost certainly represented different thermoregulatory challenges towards the maintenance of stable body temperatures, to which the animals likely responded with a broader suite of behavioural and physiological adaptations (Séon et al, 2020).

Studies have also demonstrated that, at small sizes (<5kg), active physiological mechanisms that enable heating/cooling rate hysteresis – as opposed to passive biophysical attributes such as the thermal inertia and surface-to-volume scaling - are of paramount importance for thermoregulation and maintaining Tb within an optimal set point range (Smith, 1974; Seebacher et al, 1999). In particular, cutaneous blood flow enhances thermal exchange rates and general thermoregulation of crocodilians of any size. Increased perfusion to peripheral tissues (Lang, 1987), such as the skin and limbs, as well as to highly vascularised osteoderms (Clarac & Quilhac, 2019), facilitates heat dissipation when body temperatures are high, while improving heat acquisition when body temperatures are low. Research on the American alligator (*Alligator mississippiensis*) has demonstrated that variations in cutaneous blood flow contribute to rapid temperature changes, reflecting an adaptive response that aids in precise thermoregulation without the need for extensive movement (Smith, 1976; Smith et al, 1978).

Therefore, considering the cutaneous circulatory system as a primary route of thermal exchange with the environment, the subsequent redistribution of blood - and hence, of heat - within the body is another critical mechanism by which crocodilians manage their internal temperatures. Studies on the saltwater crocodile (*Crocodylus porosus*) suggest that changes in blood flow — particularly through peripheral and cutaneous routes — allow for efficient temperature control in response to environmental shifts (Seebacher & Franklin, 2007). This process of selective blood redistribution appears to be tightly regulated, allowing crocodilians to maintain optimal temperatures even during extended periods of basking or immersion (Smith et al, 1978).

Furthermore, the same mechanism may result in varying degrees of regional heterothermy. This is a pattern, described by Heath (1964) and widely acknowledged in smaller ectotherms such as lizards (e.g. Sannolo et al, 2014; Barroso et al, 2016) and snakes (e.g. Cox et al, 2023), whereby different body parts may simultaneously be at different temperatures. This is also achieved through a combination of physiological features, such as the vasomotor system and sites of thermal exchange (Porter et al, 2016), as well as differential levels of localised thermal inertia of the different body parts (inherent from their different sizes and tissue compositions). Ultimately, it allows the animal to resort to source-sink dynamics for heat acquisition and redistribution, allowing

the temperature of certain body parts (e.g. the dorsum) to fluctuate extensively and beyond optimal (Grigg & Alchin, 1976) and then act as a prolonged (and controlled) heat source for more sensitive regions (e.g. the head/brain; Johnson, 1974; Sannolo et al, 2014; Porter et al, 2016), all modulated by a tight cardiovascular control (Heath, 1966; Grigg & Alchin, 1976). Such cardiovascular control mechanisms thus enhance the effect of thermoregulatory behaviours, highlighting the sophistication of the interplay between behavioural and physiological adaptations.

Altogether, these findings suggest that crocodilians heavily rely on finely tuned physiological responses as much as on behavioural regulation, allowing them to thrive in thermally diverse environments. Furthermore, the level of reliance on such physiological and/or behavioural mechanisms may change between species and throughout an animal's ontogeny since, as it grows larger, it is increasingly able to capitalise on the passive biophysical advantages of its large size.

Such complex interactions meant that the study of crocodilian thermoregulation has attracted extensive interest. Nonetheless, research has mostly been limited by the invasiveness and logistical constraints for obtaining precise, while biologically meaningful, body temperature measurements, particularly in natural contexts. Early research relied heavily on invasive techniques, such as probes (e.g Smith, 1975; Grigg & Alchin, 1976) or surgically implanted (often intraperitoneally) data loggers/transmitters (e.g. Diefenbach, 1975; Seebacher, 1999; Bassetti et al, 2014), to obtain [core] body temperatures. While these methods provide valuable data on internal temperature dynamics, they pose significant limitations, including potentially altering natural behaviours and increasing stress (Langkilde & Shine, 2006). Furthermore, these methods are labour-intensive and often impractical for long-term monitoring in natural settings (Grigg & Kirshner, 2015).

The development of radio-telemetry in the latter half of the 20th century enhanced the interest in surgically implanted transmitters as these allowed for more continuous temperature monitoring in free-ranging crocodilians (e.g. Johnson et al, 1976; Seebacher et al, 1999; Hocutt, 2022). Nevertheless, the requirement for surgical implantation remained a major drawback. Veterinary expertise, often beyond the skills of most field biologists, as well as some level of analgesia or anaesthesia needed (depending on the procedure), involve additional resources and risks, especially for ectothermic animals (Chatigny et al, 2017). Such drugs may also disrupt the animal's physiology and/or behaviour, even if only for a short period of time (Mosley & Mosley, 2015), thus potentially influencing the temperatures measured. Hence, there is a growing demand for less

invasive, more sustainable techniques that can capture ecologically relevant temperature data with minimal impact on the animals.

Fortunately, recent advances in technology provide new tools for measuring body temperatures, including non-invasive approaches such as infrared thermography (IRT) (Tattersall, 2016) and minimally invasive instruments like small-sized temperature-sensitive transponders and dataloggers (e.g. Roark & Dorcas, 2000; Langer & Fietz, 2014; Whitaker & Srinivasan, 2018). IRT offers the potential to capture surface temperatures without physical contact, using thermal images to infer physiological states, such as internal temperature (e.g. Luna et al, 2013; Burns et al, 2015) or blood flow patterns (e.g. Clarac & Quilhac, 2019), through external temperature readings. However, studies on reptiles and some endotherms suggest that, while IRT can be effective in inferring internal state, it require careful calibrations as surface temperatures do not always mirror core body temperatures (Langer & Fietz, 2014; Barroso et al, 2016; Tattersall, 2016). Furthermore, patterns of regional heterothermy become of paramount importance when selecting the appropriate location for inferring internal Tb. For example, studies on small terrestrial lizards have shown that certain body regions, particularly the eyes, can serve as reliable proxies for internal temperatures, provided the appropriate species-specific calibrations are performed (Barroso et al, 2016, 2020, Mochales-Riaño et al., 2025). Nevertheless, this has yet to be validated in larger and/or semi-aquatic counterparts like crocodilians. Amphibious lifestyle poses additional challenges, as immersion (or skin evaporation when emerged) can rapidly alter surface temperatures (Tattersall et al, 2006; Tattersall, 2016) while trigger physiological responses modifying cutaneous blood flow (and heat distribution) (Seebacher, 1999), affecting the magnitude of surface-to-core gradients and potentially leading to incorrect estimations of internal state.

On the other hand, temperature-sensitive transponders and data-loggers represent a more recent innovation, offering a minimally invasive method for internal temperature monitoring that can be used in the field without the need for frequent handling. These devices - many already successfully implemented in small mammals and reptiles - can be implanted subcutaneously (e.g. Whitaker & Srinivasan, 2018), intraperitoneally (e.g. Bassetti et al, 2014), attached to the skin (e.g. Langer & Fietz, 2014), or even administered orally (Seebacher et al, 1999), and allow for continuous, sometimes autonomous (i.e. data-loggers), internal temperature tracking. For crocodilians, deploying these solutions at a more superficial (e.g. subcutaneous or intramuscular) level could serve as a compromise between invasive core measurements and immediate

environment or operative temperature readings, also providing insights into thermal gradients across the body with minimal disturbance even in free-ranging animals. However, proper guidelines and calibrations must be defined, while environmental factors, such as immersion in water or exposure to sun, must be carefully considered when pondering the adequate design (see review by Franklin et al, 2009), placement and calibration of such devices in order to ensure reliable inferences of internal state, especially under variable field conditions.

The current study tests a combination of classical and novel measurement tools to determine their reliability and comparability as proxies for internal body temperature. Namely, we fitted wild and captive *Crocodylus moreletii* with a suite of temperature sensors (probes, loggers and transponders) across multiple body regions to capture a comprehensive thermal profile. We employed an array of custom-built subcutaneous and surface temperature loggers and transponders in various body regions of several animals of a wide size range. These sensors allowed assessing thermal gradients across different body regions and evaluating their relationship with core temperature benchmarks such as cloacal (Diefenbach, 1975) and stomachal (Seebacher et al, 1999). Furthermore, infrared thermography was also used to obtain temperature readings from the eye (following previous work on lizards – Barroso et al, 2016 & 2020). Ultimately, this study aims to contribute with quantitative evidence regarding the practical use of modern, less invasive tools to measure body temperatures in crocodilians. Additionally, we aim to suggest easily measurable, reliable proxies of internal body temperature, ultimately with the hope of furthering the knowledge in crocodilian thermoregulation, while resorting to up-to-date tools and methods that minimise disturbance to the studied subjects.

4.3. Materials and Methods

4.3.1 Sampling and Animal Handling

Three experiments, taking place over two consecutive field seasons (June to August of 2021 and 2022), were conducted on Morelet's crocodiles *Crocodylus moreletii* Duméril & Bibron, 1851. The experiments consisted of comparisons between 1) thermography-measured eye temperature and contact-measured cloacal temperature (2021 and 2022); 2) subcutaneous dorsal temperature and cloacal temperature (2021); and 3) multiple internal, external and subcutaneous body temperatures (2022). While experiments 1) and 3) relied on data collected from both wild and captive animals, experiment 2) was performed solely on the latter.

Data collection on captive animals took place *in situ,* on captive-born animals, at the "Biosistemas Productivos Cocodrilo" crocodile farm (hereafter "[BPC] farm"), a registered "Unidad de Manejo para la Conservación de la Vida Silvestre", in Campeche, Mexico, within *C. moreletii's* native range (Fig. 4.1). In this farm, a breeding population (n≈60) of *C. moreletii* is maintained in a large outdoor enclosure (80x40m, circa 1:2 water:land ratio). The hatchlings, juveniles and subadult animals are kept in a range of differently-sized, communal (hatchlings and juveniles) or individual (subadults) water/land tubs or cement enclosures. Enclosures are all outdoor, under natural environmental conditions and provide sufficient habitat heterogeneity for a range of thermoregulatory behaviours (González-Jáuregui, pers. obs.).

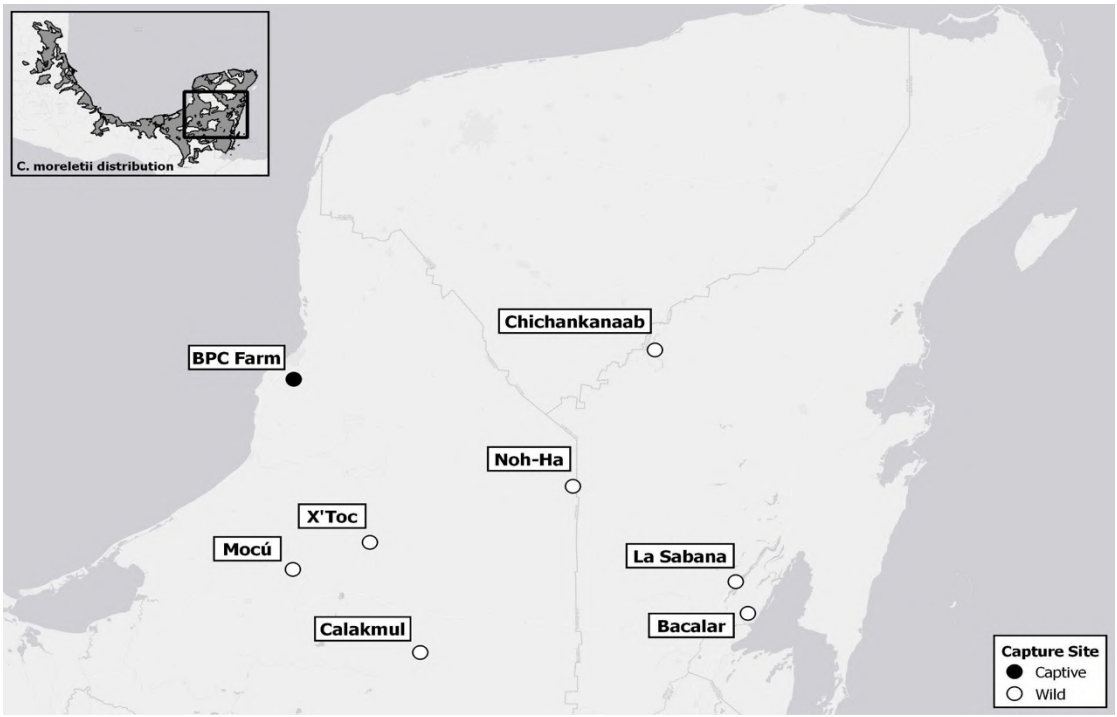

Fig. 4.1: Map of the Yucatan Peninsula (SE Mexico) showing the location of the sampling sites for wild (white circles) and captive animals (black circle representing the "Biosistemas Productivos Cocodrilo" farm). Inset showing the distribution of C. moreletii (Platt et al., 2023) in dark grey.

Wild crocodiles, however, were sampled in several sites across the Yucatan Peninsula, Mexico (Fig. 4.1). These locations encompass a representation of habitats (e.g. "aguadas"/ponds, lakes and lagoons) occupied by this species, subject to multiple levels of disturbance and protection. Crocodiles surveys were carried at night by approaching individuals either by boat, from the shore or by wading in shallow water. Captures were performed by noosing it around its neck with a pole with a break-away noose and

crocodiles were restrained using a pole-snare (Ketch-All Animal Restraining Pole), ropes and tapes (Vliet, 2014; Sánchez Herrera et al, 2011). Animals were subsequently immobilised with rope, the metal noose removed, and the jaws secured with tape for the duration of the data collection process.

With the animal restrained, a series of morphological traits were measured including: snout-to-vent length (SVL; ventrally and to the start of the cloaca), total length (TTL), maximum abdominal circumference, head length and [maximum] head width (Sánchez Herrera et al, 2011; Hutton, 1987), all with a tape measure to the next 1 cm, as well as mass (albeit only for animals <50kg), with spring balance (PESOLA® spring scales of 5 kg - precision: 0.05kg; 10 kg - precision: 0.05kg; and generic electronic scale of 100 kg - precision: 0.1kg). The same measurements were taken the same way from wild and captive animals and for all three experiments.

Additional samples and measurements from wild crocodiles were also collected for ongoing parallel CRMC projects in the Mexican Yucatan Peninsula (for more information see https://www.crocodileresearch.com/projects), and each animal was individually marked with a numbered interdigital tag, as part of a national crocodile population monitoring programme (Sánchez Herrera et al, 2011). This synergistic data collection, whereby multiple projects sampled from the same animals, maximises the amount of useful data collected per individual while minimising animal and logistic costs.

Captures and the subsequent data collection were made overnight and the entire process performed in the field, *in situ.* Animals were restrained for the minimum amount of time required for data collection, after which they were released within the same water body where they were caught. As for all three experiments in this study, all captures, handling and measurement procedures were performed by experienced personnel in order to minimise stress and risk to both the crocodiles and the handlers. All field activities were performed in compliance with the guidelines for use of live amphibians and reptiles in field and laboratory research (Beaupre et al. 2004)

Research permits for capturing the animals and sample collection for this study were issued by the Dirección General de Vida Silvestre (DGVS) of the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) of Mexico (SGPA/DGVS/03336/22). The surgical procedures used throughout this study were devised, tested and overseen by a veterinarian

4.3.2 Calibrating Thermography-Measured Eye Temperature (Experiment 1)

The set-up of temperature data collection varied depending on whether the animal was from the wild (in the field, regardless of size), captive (in the farm) and small (SVL<50cm) or captive and medium-sized (SVL range: 50-90cm). For most individuals, temperature data collection was repeated across several time points. Hence, the variations in protocol were to account for the practicality of obtaining multiple, realistic (i.e. temporally independent) temperature measurements of animals of different sizes and from different contexts (farm vs wild).

For wild animals, the first (and often only) time-point of temperature collection happened immediately upon capture. As soon as the animal was secured (<3 mins), a thermal photo of the eye of the animal was taken using a radiometric camera (FLIR Lepton 2.5 LWIR micro thermal camera module; FOV: 50º, sensitivity: 0.050 °C; accuracy: ± 5%; IR image resolution: 80×60 pixels; Flir Systems Inc., Wilsonville, Oregon, USA) integrated on a CAT S61 phone (Bullitt Mobile Ltd, Caterpillar Inc. Illinois, USA). Distance between the camera and the subject (i.e. eye of the crocodile) was kept to a minimum (circa 15-20cm) to avoid biases such as pixel size effects and attenuation from the environment, (Faye et al, 2016), and remained the same for all measurements either in wild or captive individuals.

Immediately (<1min) after taking the thermal photo, the cloacal temperature (T_c) of the animal was measured with a contact thermometer (Hibok 14, precision: 0.1ºC, accuracy: 0.3%) fitted with a thin, k-type thermocouple probe. As common practice in reptile thermal ecology studies (Taylor et al, 2020), the probe used had a smooth, rounded tip to avoid potential injury to the animal. The same thermometer was also used to measure water, air and ground (if applicable) temperatures at the time and exact location where the animal was caught.

Subsequently to the eye and cloacal temperature readings, the animals were brought to land for further data collection, including morphometric measurements. Whenever animals were kept on land for longer (>1h) periods without being directly handled (e.g. due to a backlog of multiple animals to process), then multiple eye and cloacal temperature measurements were taken on the same individual, with a minimum interval of 30 minutes between repeated measures. For captive animals, a more strict criterion was employed, however, due to the large range of sizes of animals available, two size-adjusted enclosures were used for this experiment.

The set-up for younger (1-3 years old), and smaller (SVL < 50cm) captive animals (n=20; Fig. 4.2), consisted of a large plastic tub (185Lx95Wx50H cm) filled with 15cm of water. A 85x40cm plastic board was placed centrally in the tub, supported just above water level by a brick. This provided a dry basking platform above it as well as an underwater, shaded refuge below. The tub was placed outdoors, half in shade and half in direct sunlight, and the water was made to vary (mean = 28.6 ºC, range: 22.3-33.5ºC) by the regular addition of (~1-5 litre) blocks of ice. These conditions created thermal heterogeneity in the habitat, thus promoting thermoregulatory behaviours (e.g. basking, shuttling) in the animals.

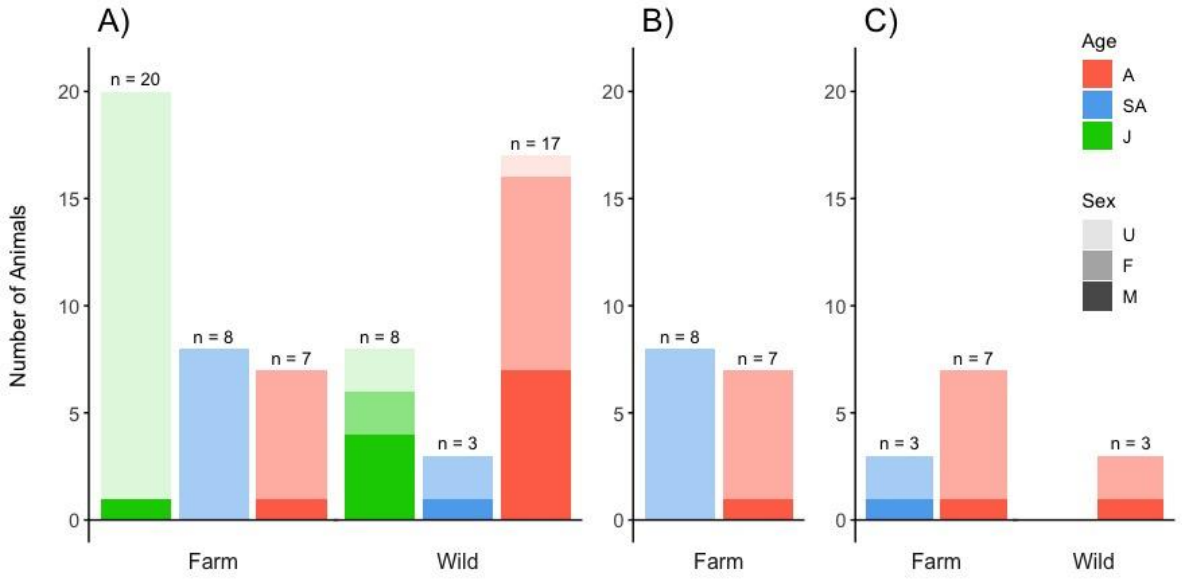

Fig. 4.2: Number of wild and captive animals, divided per sex (shade - male, female, undetermined/unknown) and age class (colour - green = juvenile, blue = sub-adult, red = adult) tested in A) Experiment 1, B) Experiment 2, and C) Experiment 3. The numbers above each bar represent the total sample size for that age class.

Since the objective was to generate a wide range of body temperatures, and not to estimate thermal physiology parameters or behaviours, potential effects of competition or interactions between individuals were irrelevant and hence multiple animals could be tested at the same time. Thus, for each trial, 2 to 3 animals were placed simultaneously in the enclosure. Temporary markings were made in each animal with an inert wax marker (Paintstick® marker, Markal®, USA), uniquely identifying each individual. The SVL, TTL, maximum circumference and mass of each animal was measured prior to their trial. Additionally, to facilitate recapture, animals were fitted with a long (~1-1.5m) leash around their chest, made out of a light string. Between captures, the leash was allowed to trail behind the otherwise unrestrained (e.g. mouth untapped) animals.

Each trial had a duration of 3 hours and ran in the time period between 10:00h and 16:00h. Every 30 minutes, each individual was caught and its eye and cloacal temperatures measured immediately upon capture (<1min between measurements of each body part) with the thermal camera and contact thermometer respectively and as described previously for data collection in wild animals. When handling the crocodiles, care was taken to avoid touching the eye or general head region as well as the start of the tail, in order not to influence the eye and cloacal temperature measurements - the leash aided in this. The animal's mouth was left untapped throughout the entire process to allow thermoregulatory behaviours of the head such as gaping and panting. Furthermore, the temperatures of the air, water and of the basking platform were also measured, with the same thermometer.

For the medium-sized (SVL range: 50-90cm) animals (n=15; Fig. 4.2), the set-up consisted of an outdoor fenced enclosure (6m diameter) with bare soil and leaf litter as substrate. Dug into the centre of the enclosure, a water-filled plastic tub (290Lx80Wx45D cm; ~1000 litres) functioned as a pond. A large tree adjacent to the fenced area provided patches of shade across the enclosure, which varied in extent (50-90%) depending on the time of day, thus generating thermal heterogeneity to the environment. As for the previous set-up, this induced thermoregulatory behaviours in the test animals, which were further encouraged to shuttle between land and water by regular additions of large (~15-50 litres) ice blocks to the pond. The latter aided in maintaining a broad, yet realistic, array of ambient temperatures for the crocodiles to utilise.

The enclosure's thermal availability was monitored throughout the entire experimental period (day and night, on and off-trials) with environmental temperature loggers (Onset HOBO Pendant Waterproof Temperature & Light Intensity Logger - 8kB memory, precision: 0.14ºC, accuracy: 0.53ºC). These were placed in three locations inside the enclosure (full sun, permanent shade, inside the water tub - on the shaded side) and registered ambient temperature every 15 minutes. An additional k-type thermocouple probe was placed on the opposite [sunnier] end of the water tub to account for potential temperature gradients inside the water. The same contact thermometer used throughout the study was employed to measure such temperature with the same periodicity as that of the data loggers (15mins).

Prior to being placed inside the test enclosure, animals were fitted with a rope harness around their chest and front legs, with a long trailing end that extended beyond the fence of the enclosure. This ad-hoc leash allowed the animals to be manipulated from outside

of the fence and without restraining their heads or tape their mouths. The leash was long enough to allow animals to move across the entirety of the enclosed area.

In addition to the leash, a long (15m) yet thin (1-2mm, with rounded tip) k-type thermocouple probe was inserted <2cm into the cloaca of the crocodile (Fig. 4.3) and connected to the thermometer (Hibok 14, precision: 0.1ºC, accuracy: 0.3%) also outside the fenced enclosure. Inert and non-toxic cyanoacrylate glue was used to attach a section of the probe's to the scales at the base of the crocodile's tail, just anterior to the cloaca. These were previously cleaned with water and 96% ethanol for better adhesion. Strong, water-resistant medical adhesive aided securing the probe in place, and the whole area was then enveloped in duct-tape loops around the animal's tail, covering the attachment site just below the cloaca. Special caution was taken to ensure that the cloaca itself was left unobstructed, and that the duct tape loops were not restricting blood flow to the tail. This arrangement was usually sufficient to maintain the tip of the probe inside the crocodile's cloaca throughout the course of the entire trial. Nonetheless the position of the probe was checked on a regular basis during the experiment and adjusted when needed. At the end of the trial, the probe was easily detached by cutting off the duct-tape and peeling off the medical adhesive. The attachment site was once again cleaned (ethanol then water) leaving the animal unharmed or without any trace of the procedure.

With the attached leash and cloacal probe, the crocodile was then allowed to freely roam in the test enclosure. Periodically (every 15 minutes), the leash was used to briefly pull the animal towards the enclusure's fence from where a thermal photo of the eye was taken using the thermal camera mentioned prior. The photo was taken through the holes of the fence in order to allow a small distance (15-20cm, as done with the wild crocodiles) between the camera and the animal, while protecting the researcher from the unsecured mouth of the crocodile. Immediately after, a reading of the cloacal temperature was also obtained. After collecting this data (for experiment 1 and 2 - see below) the leash was slacked and the animal allowed to once again roam free in the enclosure. It was additionally noted whether the animal was on land vs in water, and in the sun vs in the shade prior to the measurements, and a soil temperature reading from the nearest location to the animal's initial position, was taken with the same thermometer (and similar k-type thermocouple probe).

Each animal was tested once and independently (i.e. only one animal in the test enclosure). Tests took place between 9:00h and 18:30h, and lasted between 3-4h for

each animal. After each trial every animal was returned to its original enclosure (but see Experiment 2 below).

4.3.3 Subcutaneous Dorsal Temperature (Experiment 2)

This experiment ran concurrently, with the same duration, and on the same medium-sized (SVL range: 50-90cm, n=15; Fig. 4.2) animals, as Experiment 1. When preparing the animals for Experiment 1 (i.e. recording morphometrics and attaching the harness and cloacal probe), the crocodiles were also fitted with an *ad hoc* subcutaneous temperature transponder (Fig. 4.3). A small surgery was required in order to implant the thin (diameter: 22mm, thickness: 2.5mm) transponder below a large scute from the animal's mid-dorsum area. An off-centred scute was chosen in order to avoid the area directly above the spine (Fig. 4.3). To expedite the procedure, and hence minimise its invasiveness, the scute chosen was large enough for entirely accommodating the transponder, without the need to cut into adjacent ones. Thus, the procedure was only considered for animals larger than 50cm SVL.

Prior to the small surgery, the chosen scute and surrounding area were brushed with soap and water to remove encrusted debris, and then disinfected with 96% ethanol. A small (<2cm) incision on the skin adjacent to the chosen scute was made with a scalpel (handle nr: 4, blade nr: 21). The adipose and connective tissue between the skin/scute were separated from muscle below using the scalpel, thus making space for the device. Finally, the transponder was inserted into the space opened underneath the scute, and the incision then temporarily closed (for the duration of the trial) with non-toxic cyanoacrylate glue and medical tape.

After the surgery and all the preliminaries described for Experiment 1, the animal was released in the test enclosure. To obtain temperature readings, the transponder was read, via a near-field communication protocol (NFC), by an app (GATTOR v1.0, ElectricBlue, Portugal) on the CAT S61 phone. For this purpose, however, the phone and the temperature transponder (hereafter NFC transponder) needed to be in very close (<2cm) proximity. Hence, whenever the animal was being restrained for collecting the data for Experiment 1 (every 15 minutes), the phone was placed in contact with the animal's point of incision and the reading of subcutaneous dorsal temperature obtained this way. The reading was attained within a couple of seconds of touching the dorsum of the animal, hence, the restraining time was kept to a minimum.

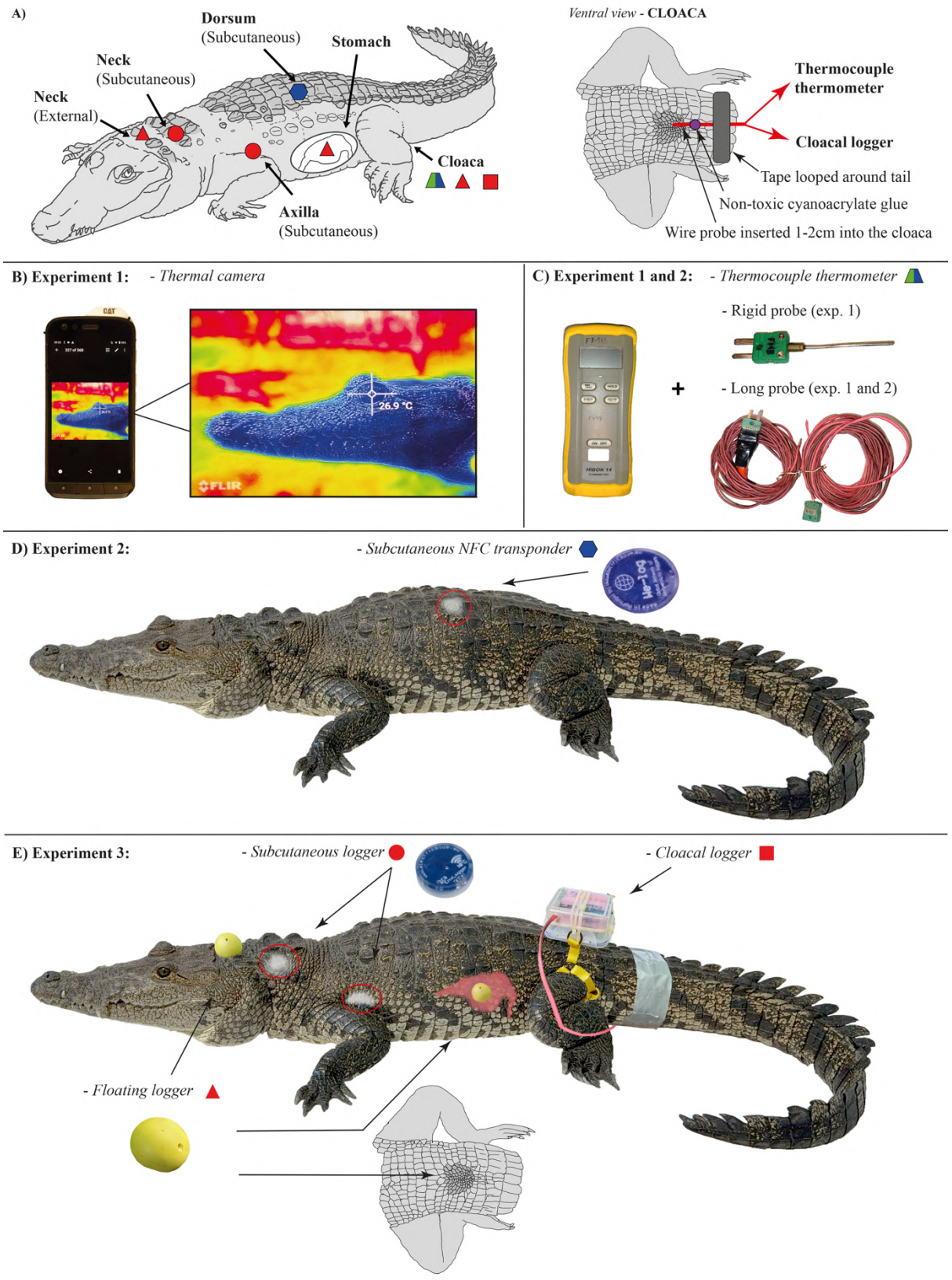

Fig. 4.3: Diagram of the several types of temperature sensors, loggers and transponders used for all three experiments, and indication of their placement location. Image credit: Giulia Simbula

Furthermore, the close temporal proximity (<2mins) between the cloacal temperature reading (Experiment 1) and that of the dorsal temperature recorded from the NFC sensor meant that these could be assumed as paired, as the measurements done for the eye temperature calibration in Experiment 1. The same applied to the remaining environmental temperature readings collected then, as well as for the information regarding the animals use of the habitat in the moments prior to the reading (see Experiment 1 above).

At the end of this trial, the transponder was removed by reopening the incision, retrieving it, and then permanently closing off the incision with cyanoacrylate glue. Finally, the surgical area was sprayed with a topical antimicrobial that promotes wound healing (Aluspray®, Vetoquinol S.A., France) before releasing the animal back to its original enclosure. The entire process was relatively fast (<15 mins) and any bleeding during the surgeries was minimal and easily contained by applying pressure with sterile gauze and haemostatic forceps. Additionally, no postoperative bleeding or further complications were observed as all animals made a full recovery from the procedure leaving any scarring or signs of the incision completely indistinguishable within less than a year (Barroso & González-Jáuregui, pers. obs.).

4.3.4 Multiple, Simultaneous Body Temperatures (Experiment 3)

Data collection for this experiment took place one year after completing Experiment 2 and having preliminarily analysed such data. Hence, preliminary data analysis allowed the initial protocol to be optimised towards maximising the data collected while minimising invasiveness and disturbance. This involved a redesign of the tools to measure body temperatures to allow a more autonomous (i.e. less human-crocodile interaction), less restrictive (i.e. no leash) and more flexible (i.e. applicable to different body parts) temperature recording. Hence, three types of ad hoc loggers (i.e. that both measure and store temperature) were developed (Fig. 4.3): the cloacal probe logger, the subcutaneous logger, and the floating logger. Furthermore, in addition to the HOBO environmental data loggers used in Experiments 1 and 2, three additional temperature data loggers (model: EnvLogger T2.4, accuracy ≤ 0.2ºC, precision ≤ 0.1ºC, ElectricBlue, Portugal) were added to the main experimental enclosure. One replaced the thermocouple probe in the water, and the other two provided replicates of shade and areas of direct sun temperatures within the enclosure. As before, these loggers

registered environmental temperatures every 15 minutes throughout the course of the entire experiment period.

The cloacal probe logger (hereafter cloacal logger) was based on a ESP32 microcontroller (ESP32-WROOM-32D, Espressif Systems Co. Ltd, Shanghai, China) unit, two lithium ion batteries (in series, each with 3200mAh capacity) and a NTC thermistor probe (resistance = 10kΩ, accuracy = 1%, range = -55ºC to 125ºC, τ = 15s; length of probe cable: 90cm, diameter of probe tip: 0.34cm). The battery unit was encased in epoxy resin for protection and to provide negative buoyancy to the otherwise positively buoyant and watertight plastic casing, which additionally protected the battery and the microcontroller unit (Fig. 4.3, Table 4.1). The NTC probe cable was enveloped in a flexible plastic sheath making the entire system impermeable to water, as confirmed by preliminary tests during the R&D stage (Barroso & Barroso, pers. obs.). The logger's sampling frequency was configured using an app (Serial Bluetooth Terminal v1.38) installed on the CAT S61 phone, with which it communicated via Bluetooth.

As for the subcutaneous and floating loggers, they are adaptations of the T2.4 EnvLogger described above, sharing the same specifications albeit with a smaller battery (CR1220, 3V lithium-ion battery). The latter was a compromise to reduce the size and mass of the logger, making it easier to surgically implant or to float in water, respectively and depending on the logger type and objective. This subcutaneous logger was smaller in diameter (16mm) but thicker (5.8mm) than the NFC transponder used in Experiment 2 (Fig. 4.3, Table 4.1), but was equally encased in the same inert epoxy resin (HB Eposurf 2 A+B, HB Química, Portugal). The floating logger was also encased in this epoxy but mixed in with glass micro-beads which provided the desired positive buoyancy. Additionally, this logger was dome-shaped (diameter of the base: 20mm, maximum diameter: 26mm, height: 21mm), bright yellow (for increased visibility when retrieving it after it self-detaches) and perforated (one hole of diameter = 2.5mm) from one side to the other close, to the top of the dome, to allow a string to be run through it (see Fig. 4.3, Table 4.1). These two loggers, as well as the EnvLoggers, were configurable (e.g. frequency of temperature measurement) and the stored data downloadable through the EnvLogger app (EnvLogger Viewer v5.7, ElectricBlue, Portugal) on the same phone, communicating via NFC.

	NFC Transponder	Subcutaneous Logger	Floating Logger	Cloacal Logger	Thermocouple Thermometer
Precision (ºC)	0.1	0.1	0.1	0.1	0.1
Accuracy (± %)	0.2	0.2	0.2	1.0	0.3
Range (ºC)	-40 to 85	-40 to 85	-40 to 85	-55 to 125	-50 to 1300
Dimensions (cm) Diameter = ø Length = L Width = W Height = H	Ø = 2.20 H = 0.25	$Ø_{probe}$ = 1.60 H = 0.58	$Ø_{max}$ = 2.60 $Ø_{base}$ = 2.00 H = 2.10	$LxWxH_{case}$ = 10.7x8.2x4.7 L_{probe} = 90 $Ø_{probe}$ = 0.34	L_{probe} = 150 $Ø_{probe}$ ≤ 0.20
Payload* (g)	1.7	2.4	7.0	250.0	*NA*
Log vs Transmit	Transmit	Log	Log	Both	Transmit
Communication Protocol	NFC	NFC	NFC	Bluetooth®	Wired
Mode of Attachment	Surgical implantation	Surgical implantation	CA glue** Ingestion***	Harness (logger) Tape + CA glue** (probe)	Tape + CA glue** (probe)
Body Part(s) Measured	Dorsum (subc.)	Neck (subc.); Axilla (subc.)	Neck (ext.); Stomach; Cloaca****	Cloaca	Cloaca
Experiment(s)	2	3	3	3	1;2
Model; Manufacturer	(*ad hoc*); ElectricBlue[CRL], Portugal	(*ad hoc*); ElectricBlue[CRL], Portugal	(*ad hoc*); ElectricBlue[CRL], Portugal	(*ad hoc*); Gonçalo Barroso, Portugal	HIBOK 14; HIBOK, Spain

** Actual mass of tag or logger carried by the animal;*

*** Non-toxic cyanoacrylate (CA) glue used for external attachment;*

**** Forced or voluntary ingestion of the logger for measuring stomach temperature;*

***** Only deployed on animals where cloaca was large enough to insert the logger without forceful insertion required.*

Table 4.*1*: Table with general technical specifications of each of the sensors/loggers/transponder.

Crocodiles from both the BPC Farm (n=10) as well as from the wild (n=3) (Fig. 4.2) were fitted with a combination of up to 5 loggers per animal, simultaneously measuring temperatures of different body parts, namely: neck externally, neck subcutaneously, axilla subcutaneously, stomach and cloaca. For the captive animals, two test groups were distinguished based on body size categories: medium-sized (SVL: 66-81cm, n=7) and large animals (SVL: 104-131cm, n=3). Medium-sized animals were tested in the same experimental enclosure as in Experiments 1 and 2, while larger animals were tested in a larger natural enclosure (10x10m with two water tubs of diameter = 2.1m) where they are usually kept, isolated from the main pond. Wild animals were tagged in situ, and hence only animals inhabiting smaller, isolated ponds ("aguadas") with minimal vegetation were chosen in order to maximise the chances of retrieving the loggers once they self-dislodged from the animals, after 2-3 days of sampling.

Prior to fitting the logger to the animals, morphological measurements were taken, as described previously. Loggers fitted depended on the experimental groups as all the wild and the large, captive animals only received a subset of the 5 loggers (always including at least one of the internal temperature benchmarks) due to technical limitations associated with logger recovery at the end of the trials. For the wild animals, since recapturing the animal was not guaranteed, only the floating (and self-detachable) loggers were used as these would simply remain floating in the habitat after they were dislodged (neck) or expelled (cloaca).

All captive animals from the medium-sized group could be tagged with all 5 loggers. Cloacal temperature was measured with the cloacal logger which was secured to the animals by attaching the casing to a dog harness fitted to the back legs of the crocodile (Fig. 4.3), similar to what has been done for tagging juvenile gharial (Ajjim, J., 2024, pers. comm.). The probe was then inserted into the cloaca and secured with a combination of cyanoacrylate glue, medical tape and duct-tape, as described for the k-type thermocouple probe used in Experiments 1 and 2. This procedure, albeit functional, proved inadequate for larger animals as they were able to bite the protective case thus destroying the logger. Hence, for these animals, and given their larger cloacal opening, a floating logger could be used instead (Fig. 4.3). The floating logger was simply inserted into the cloaca (as per Diefenbach, 1975) where it usually remained 1-3 days, hence sufficient for the experiment. This strategy was also used in the wild animals as chances of recapturing them to remove any other type of logger were diminute.

Measuring external neck temperature also resorted to the floating logger and was applied on all animals from the three groups, although not all loggers could be recovered. The

floating logger was attached to the space in between the post-occipital scutes (Fig. 4.3) using the flat base of the logger (i.e. the location in closest proximity to the temperature sensor inside) as the contact/attachment zone. The same inert cyanoacrylate glue used for the surgeries was utilised for the attachment of this logger as trials in the captive animals had shown it to hold in place for 2-3 days, after which the adhesive power would wear off thus releasing the logger. In cases where the logger dislodged itself from the animals within less than 24hr of monitoring, the animal was briefly restrained and the logger re-attached.

For measuring the subcutaneous temperatures of the axilla and neck regions, the smaller loggers were surgically implanted, one in the thoracic side of an anterior axilla and the other under the largest nuchal scute of the neck plate (Fig. 4.3). The same procedure as that of Experiment 2 was followed, although, since the loggers had to remain in place for a longer period of time (multiple days), 2-3 sutures (1/4 polypropylene monofilament suture, ½ circle 19mm needle, Atramat®, Internacional Farmacéutica S.A de C.V, Mexico) were made prior to adding the cyanoacrylate glue. This ensured a more secure closing of the incision both during and after the trial. As before, the surgical procedure was devised by a veterinarian and no postoperative bleeding or complications were observed with all animals making a full recovery.

As for the stomach temperature, this resorted to the floating logger (Fig. 4.3) and was only measured in captive animals due to the complexity involved in recovering the logger. For the large animals, the logger was simply inserted into a fish carcass and fed to animals accustomed to the fish-based diet. The rounded shape and inert composition of the logger meant that it would potentially resemble a gastrolith (Takasaki & Kobayashi, 2020) and hence could stay in the stomach for an extended amount of time without any harm to the animal (Seebacher et al, 1999; Brien et al, 2014; Grigg & Kirshner, 2015). Eventually, it would simply pass through the animal's digestive system and be defecated among the faeces. However, we were expecting that this could take from days to months, and hence was only practical for large, captive animals that could be monitored regularly for prolonged periods of time. Nevertheless, if such was not the case and the logger remained in the stomach for too long a time, then a stomach lavage could be performed to retrieve it.

This approach, however, was not viable for medium-sized animals due to the increased risk of intestinal occlusion. Instead, the restrained animal was prepared as if for a stomach lavage (following Taylor et al, 1978; and Gonzalez-Jauregui et al, 2019) with a size-appropriate, lubricated tube (of an internal diameter larger than the floating logger's

dimension) inserted through the oesophagus all the way to the stomach. A floating logger was fitted with a length of nylon fishing wire by looping it through the hole in the top of the logger. The loop was tied and any sharp edges from the knot melted until smooth. The logger was then fed, through the tube, into the stomach. The other end of the fishing wire was looped and tied around the rostrum of the animal.

Caution was taken to leave enough length of wire for the logger to float around freely in the stomach. It was also ensured that the size and tension (if any) of the wire would not affect the functioning of the palatal valve. This wire prevented the logger to pass from the stomach to the intestine, as well as enabled recovery of the logger at the end of the trial. The latter was achieved by performing another stomach lavage (Taylor et al, 1978, Gonzalez-Jauregui et al, 2019) during which nylon string attached to the logger was gently pulled. In all cases the logger was smaller than the normal size of the prey items (fish) normally fed to the crocodiles. Furthermore, the animals showed no impacts of the stomach lavage processes and readily resumed feeding after the trial.

All loggers were set to obtain a temperature reading every 15 minutes. Full synchronicity of the measurements was obtained by resorting to the “Pretty Dates” function of the EnvLogger app. This triggered the start of the mission to match the nearest exact quarter of the hour. The Data of each logger was downloaded at the end of each trial lasting one to three days for medium-sized animals, one day to several weeks/months for the large captive animals, or one week for the wild animals (but see “Statistical Analysis”).

4.3.5 Statistical Analysis

For all experiments, data cleaned to account for occasional, temporary dislodgement of some of the loggers (e.g. cloacal logger/probe, neck external logger), which resulted in incomplete body temperature datasets, albeit for short periods within the sampling times. However, for Experiment 3 in particular, the large discrepancy in the sampling period of the medium-sized captive animals (1-3 days) versus that of the captive large (one to several weeks) and wild (around 1 week) animals tagged, meant further trimming was necessary to address this bias. Hence, data was cropped into the portions (circa 150 time points representing around 1.5 days of data) which minimised the amount of continuous missing data on body and environmental temperatures. Furthermore, since sample size of the medium-sized animals in this experiment doubled that for the large, captive animals, two data blocks (150 time points each) per individual were selected from the underrepresented larger-sized animals group.

Additionally, since obtaining the mass of the larger (>50kg) animals was not feasible, and hence only SVL was recorded for these animals, the relationship between these two measures was explored both graphically and through a linear regression of the logarithm of both measures. A strong relationship obtained meant that SVL alone would suffice for accurately describing the overall size of the animals and hence the incomplete mass dataset could be disregarded. This was particularly relevant when choosing the appropriate explanatory variables for modelling body temperatures, as described below. The linear regression between the logarithms of mass and SVL was fitted using the *stats* R package (v3.6.3, R Core Team 2020). Furthermore, patterns in SVL were explored graphically to confirm a wide distribution of sizes between the different experimental groups, particularly between captive and wild animals.

In order to test the relationship between the surface or subcutaneous temperatures, from different body parts, with a standard internal body temperature, such as the cloaca (Diefenbach, 1975; Taylor et al, 2021) or the stomach (Seebacher et al, 1999), we resorted to Ordinary Least Squares (hereafter "OLS") regression approach with resampling (Legendre & Legendre, 2012). This method dealt with the repeated measures design of the experiments (see Barroso et al., 2016). For each pair of surface (or subcutaneous) and internal temperatures a regression was fitted, using the *lmodel2* R package (v1.7-3, 2018). In all cases the internal body temperature was considered the independent variable as it is taken as the reference body temperature, but also for consistency when comparing slopes and intercepts of the different regressions.

As per other similar studies (e.g. Meiri, 2010; Barroso et al, 2016; 2020), OLS regression was favoured over Reduced Major Axis (RMA) regression. This was because the former analysis intrinsically assumes that the body part taken as the internal body temperature represents the reference of the animal's "true" internal temperature, with no error. Nevertheless, such an assumption is not without its shortcomings, as explained further below. However, Smith (2009) reports that slopes of OLS and RMA regressions do not differ significantly when determination coefficients are large, as is the case for many of the regressions fitted in this analysis (see "Results"), and hence the choice of OLS regression still stands.

For Experiments 1 and 2, which preceded Experiment 3, stomach temperatures were not collected and hence cloacal temperature was used as the internal temperature standard for the OLS regressions. For Experiment 3, however, since both cloacal and

stomach temperature were recorded, a preliminary analysis, with the OLS regressions considering either as the standard internal temperature, was performed to decide on which to most adequately use. Ultimately, regressions with stomachal temperature as the explanatory variable showed stronger fits (higher R^2) than those with cloacal temperature, thus solidifying the choice of the former. Still, an additional OLS regression explored the relationship between the two internal body temperatures (cloaca vs stomach).

Furthermore, while the stomachal logger was known to remain in the stomach for the entire duration of the experiment, the cloacal probe occasionally dislodged itself from the cloaca. This could have led to a larger degree of erroneous data points regarding cloacal temperature. Nevertheless, data was cleaned as described above. Hence, by using the stomachal temperature data as the reference internal temperature data, this potential bias was entirely eliminated.

From these regressions, R^2 values were compared in order to contrast the strength of the relationships. Additionally, the slopes and intercepts, namely their difference from 1 and 0 respectively were also tested (within Experiment 3). These were demonstrated by plotting the relationships.

To better explore any inherent patterns affecting the magnitude of the OLS determination coefficients, the difference between the temperature of the body parts and that of reference benchmarks was calculated with the formula: *temperature of body partx - temperature of internal body temperature benchmark,* whereby the internal body temperature reference was the cloacal temperature for Experiments 1 and 2, or the stomach temperature for experiment 3. This was calculated for each time point and plotted against time of day. The effect of other variables could then be visually inspected by fitting separate curves for whether the animal was on land or in water (hereafter “land vs water”), in sun or in shade (hereafter “sun vs shade”), etc.

Such effects were also explicitly tested through linear mixed effects (LME) models. These were fitted to determine which external variables affected the temperature of the tested body parts that showed the highest OLS determination coefficients. Hence, for Experiment 3, models were fitted for the axilla and for the external neck temperature, respectively. Dorsal temperature, from Experiment 2, was also modelled since it could be used to test the importance of more variables, sun vs shade or land vs water, that were not measured in Experiment 2. A model for eye temperature, from experiment 1, was not fitted since the OLS determination coefficient was comparatively very low (R^2 =

0.455; Table 4.2) and hence eye temperature was deemed a bad proxy of internal body temperature.

The LME models were fitted with the *lme4* package (v1.1-21; Bates et al, 2015) in R and in all cases considered individual ID as the random factor. For Experiment 3 axilla and external neck temperatures. the initial models fitted represented the additive effects of the variables *time of day, age [category]* (i.e. adult, subadult, or juvenile), *sex, SVL, average air temperature* and *water temperature*, in addition to the *animal's ID* random effect, while for the dorsal temperature, obtained from Experiment 2, the additive effect of the variables *land vs water* and *sun vs shade* were also considered. Additionally, in the latter, two interaction terms were considered relevant to test in the initial model: *land vs water* : *sun vs shade* and *land vs water : average air temperature.* Note that *average air temperature* was calculated as the mean temperature (for each time point) collected from all the environmental data loggers and thermometer measures obtained from the animal's test enclosure.

Model selection, on each of these three initial models, was performed using the *dredge* function from the *MuMIn* R package (v1.15.1; Bartoń, 2015), which computed the AICc (Akaike Information Criterion corrected for small samples) for all possible combinations of variables. Models were ranked based on their AICc and considered indistinguishable if ΔAICc < 2 according to Burnham & Anderson (2003). This resulted in a list of best models from which the most important variables affecting each body part's temperature can be inferred.

All graphs were plotted using the *ggplot2* package (v3.3.5; Wickham, 2016) in R. The datasets and R scripts used for this analysis are openly available in the Github repository https://github.com/FredericoMB/Croc_Tb_Methods.

4.4. Results

Comparing the size (e.g. SVL) ranges of animals tested in captivity (SVL range: 21.2-131cm, n = 45) to that of the wild crocodiles caught (SVL range: 25-140cm, n = 31) showed that the size range of the captive animals was indeed representative of that observed from the wild-caught crocodiles (Fig. 4.4). Nevertheless, the sizes of wild animals varied considerably across sampling sites with most locations also showing a mean SVL larger than that of the farm animals tested (Fig. 4.4 D). Furthermore, the average and range of sizes of the animals tested for Experiment 1 (SVL mean ± SE =

62.5 ± 3.73cm, range = 21.2-140cm, n = 63) differed from that of Experiments 2 (SVL mean ± SE = 72.4 ± 1.74cm, range = 58.7-85.0cm, n = 15) and 3 (SVL mean ± SE = 88.7 ± 6.41, range = 66-131cm, n = 13). Furthermore, for Experiments 2 and 3 there was a minimum size threshold (SVL > 50cm) for the animals in which the loggers could be implanted thus restricting the range of sizes, and, hence, the number of animals that could be tested.

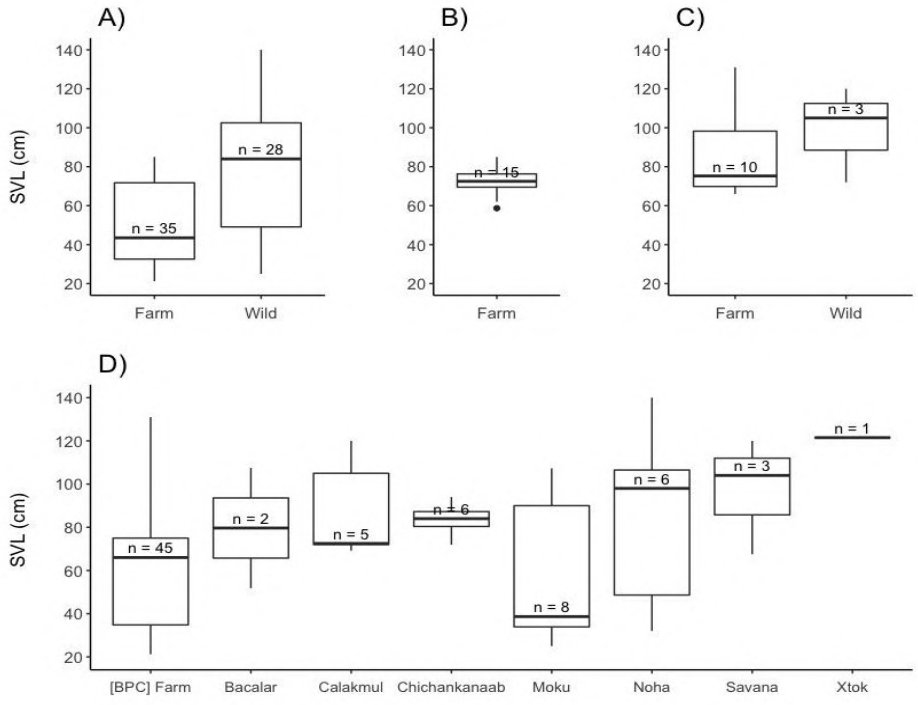

Fig. 4.4: Snout-Vent Lengths (SVL) of animals tested for A) Experiment 1, B) Experiment 2, and C) Experiment from both wild and captive ("Farm" = "BPC Farm") settings. Graph D) showing a comparison of the SVL between all the locations sampled for all three experiments, where "[BPC] Farm" encompasses all the captive animals, and all the other locations represent wild sampling sites (see Fig. 4.1). Total sample size in each location denoted by "n" within each boxplot.

Plotting the relationship between mass and SVL (Fig. 4.5 A), for animals with a mass under 50kg from which both measures were obtained, rendered the well-established pattern whereby an increase in length (SVL) results in an allometric increase in mass. The simple linear regression fitted to the logarithmic transformation of both variables (Fig. 4.5 B) showed a very strong (logarithmic) relationship between these two measures, as given by the very high R-squared obtained (R^2 = 0.988, $F_{1,63}$ = 5279, p<0.001). Assessing the fit of the regression through diagnostic plots (Fig. 4.5 C-E) confirmed that the linear model met the assumptions of heteroscedasticity and normality of residuals.

Overall, this consolidated the decision of using SVL as the sole proxy of body size of the animals for the remainder of analysis, conveniently disregarding mass, for which we had an incomplete dataset (i.e. no data for mass > 50kg). Furthermore, since both variables were highly correlated, dropping one also avoids issues associated with collinearity when fitting further linear models.

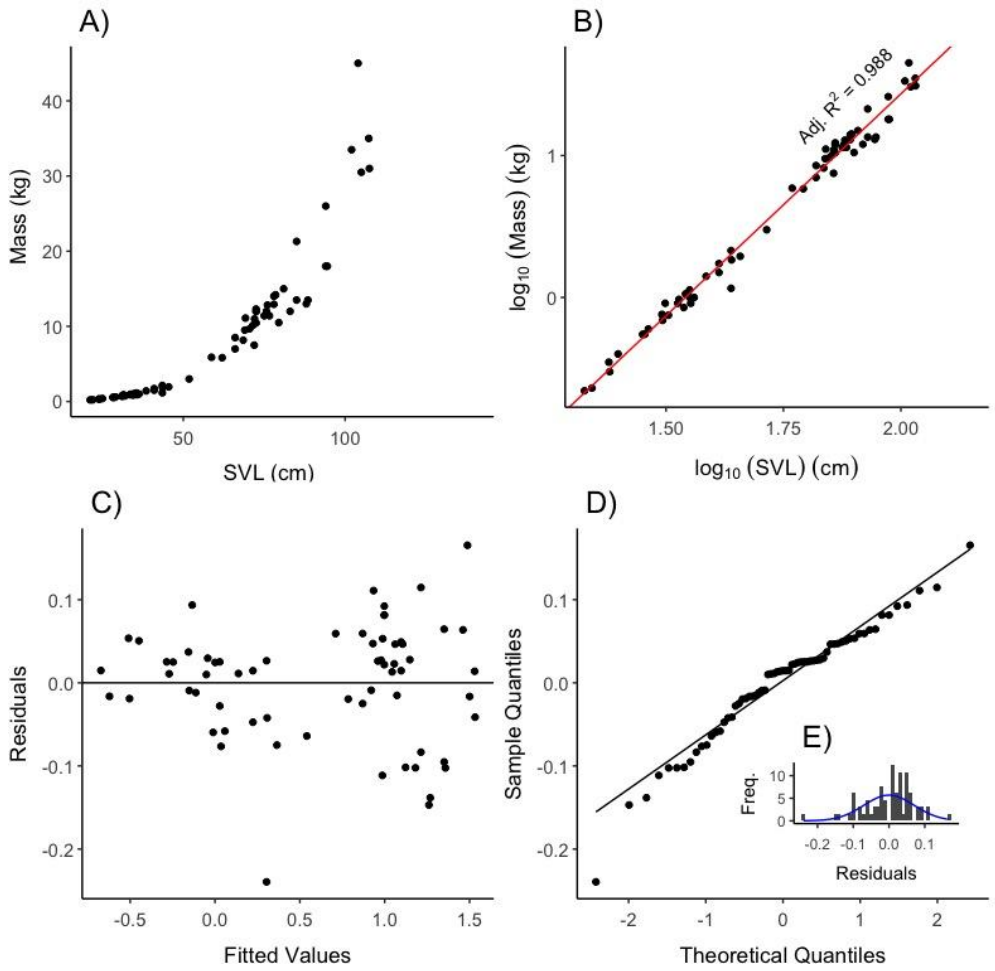

Fig. 4.5: A) shows the expected exponential relationship between mass and SVL. B) Log-transforming both variables renders a strong linear relationship (adjusted R^2 = 0.988). Diagnostic plots (C - Residuals vs Fitted Values; D - Q-Q Plot of Residuals; E - Histogram of Residuals) further validate the fit of the linear regression given the assumptions of normality (D, E) and homoscedasticity (C) of residuals are all met.

For Experiment 1, the OLS regression between contact-measured cloacal temperature and thermography-measured eye temperature resulted in a regression that significantly deviates from a linear y = x relationship (Fig. 4.6A). In other words, the slope of the line significantly differed from 1 (slope = 0.769, 95% CI's = 0.382-0.856; Table 4.2) and the intercept was considerably above zero (y-intercept = 4.207, 95% CI's = 1.654 - 6.760;

Table 4.2). Nevertheless, the determination coefficient for this regression was relatively low (R^2 = 0.455, n = 363), especially when compared to those obtained for OLS regressions from Experiments 2 and 3 (Table 4.2), which, along with the wide 95% confidence intervals (CI's) of the slope and intercept (Fig. 4.6.A; Table 4.2), hint towards and underlying variable(s) significantly affecting this relationship.

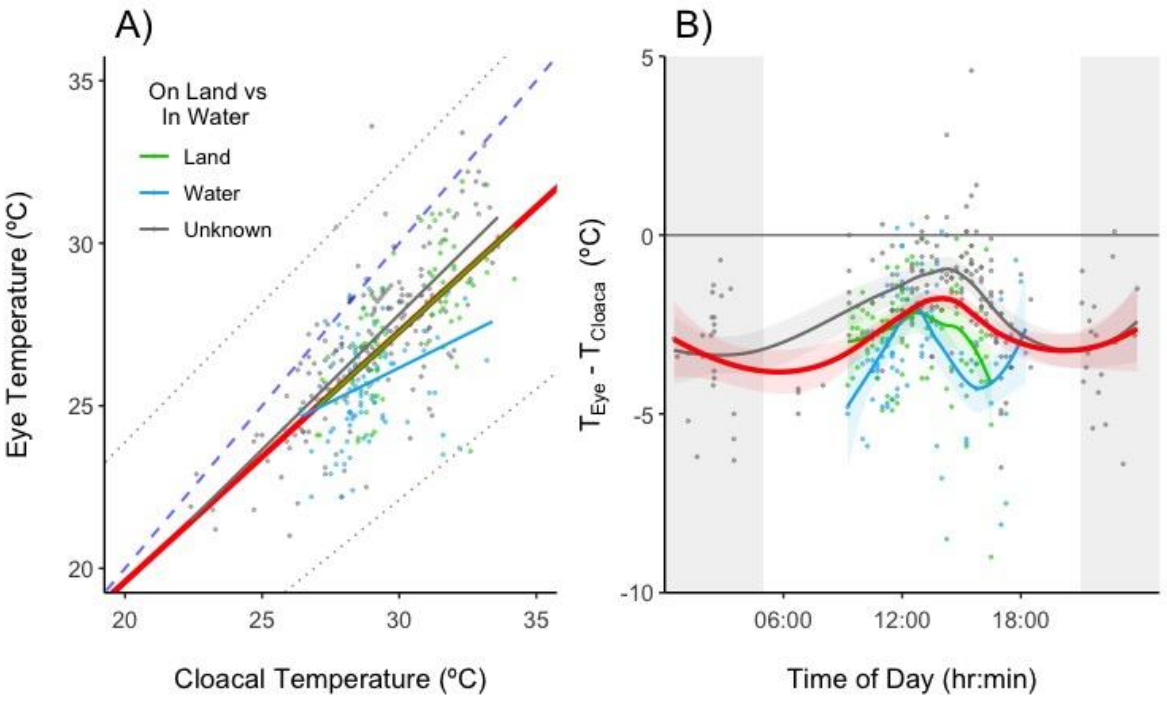

***Fig. 4.6**: Plots of the Infrared (IR) calibration. A) Shows the OLS regression lines with the red line representing the overall regression (and 95% CI's in grey, dotted lines) for the full dataset, while the light-blue, light-green and grey, full lines represent OLS regressions fitted separately according to whether the animal was found in the water, on land or unknown/unregistered respectively. Dark blue dashed line represents isometric ($y = x$) line. B) Shows how the difference between IR-measured eye temperature against contact-measured cloacal temperature varies throughout a 24hr cycle. Once again, the red line represents the general pattern (fit with a LOESS curve and SE in light-red shade) while the light-blue, light-green and grey lines represent LOESS curves fit to whether the animal was found in the water, on land or unknown/unregistered respectively. Light-grey vertical bands represent night-time hours for the sampling area (SE Mexico) during the sampling period (July).*

Indeed, when plotting separate regression lines for animals on land vs animals collected directly from the water (vs animals with no data on previous habitat), differences between regression lines emerge (Fig. 4.6.A). Namely, while the slopes and intercepts of the regressions for animals previously on land (and for animals with prior habitat unknown) closely follows that of the general OLS regression pattern, the regression line for animals in water displayed lower slope and higher y-intercept than the other two groups (Fig 4.6A). Moreover, the same pattern holds true for Experiment 2, where animals previously

on land show a steeper slope and smaller intercept (and both more similar to those of the overall OLS regression line) than that observed for animals in water (Fig. 4.7A). Finally, the importance of the *land vs water* explanatory variable is further reiterated by the fact that it is present in all (7) final best models resulting from the LME model selection for determining which variables affected dorsal temperature (Table S4.1).

Additionally, while for Experiment 1 being previously on land vs in water did not influence the temporal pattern of the difference between eye and cloacal temperature (Fig. 4.6.B), the same was not true for the difference in dorsal and cloacal temperature ($\partial T_{d\text{-}c}$) explored in Experiment 2 (Fig. 4.7B). In the latter, the ∂Td-c is larger for animals previously in the water than on land, and the temporal pattern throughout the day (from 10:00h to 19:00h) is increasingly dissimilar and opposite as the day progresses (Fig. 4.7B).

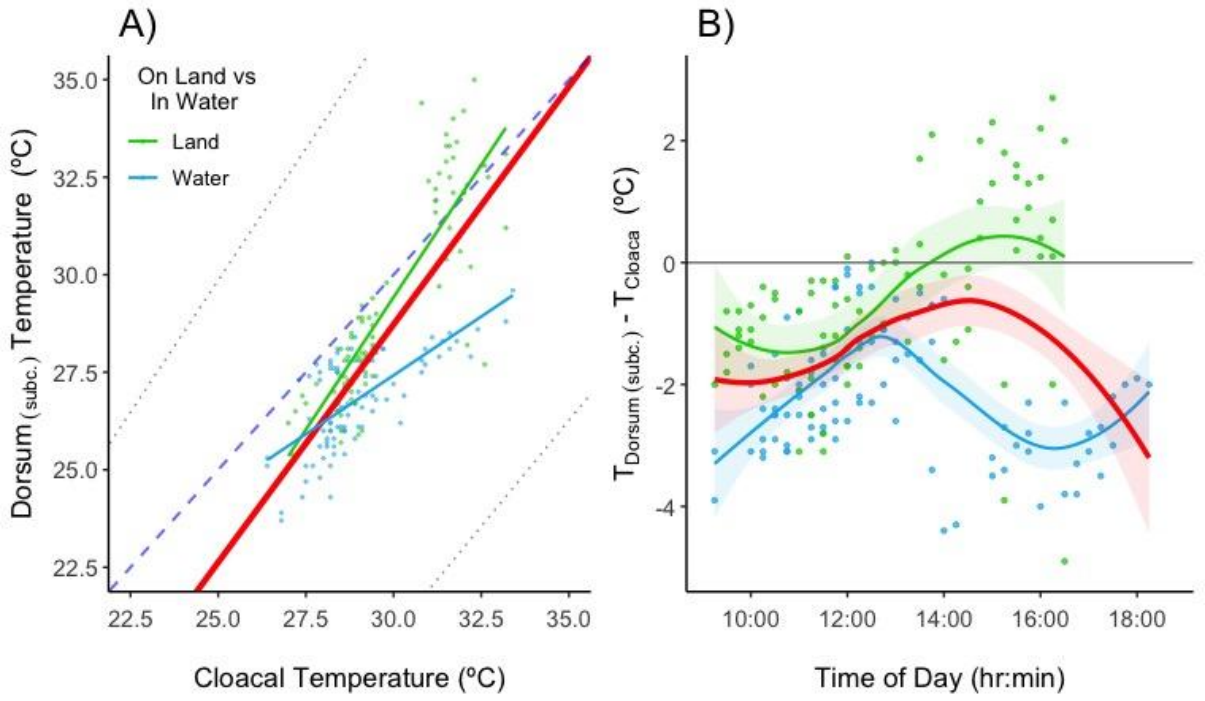

Fig. 4.7: *Plots of the subcutaneous dorsal temperature vs cloacal temperature calibration. A) Shows the OLS regression lines with the red line representing the overall regression (and 95% CI's in grey dotted lines) for the full dataset, while the light-blue and light-green full lines represent OLS regressions fitted separately according to whether the animal was found in the water, on land respectively. Dark blue dashed line represents isometric ($y = x$). B) Shows how the difference between subcutaneous dorsal temperature against cloacal temperature varies throughout the time of day and depending on whether the animal was on land (light-green) or in water (light-blue), as represented by the LOESS curves (and SE in shade of respective colour). Red line represents the overall pattern across the day.*

All other variables affecting dorsal temperature are outlined in Table S4.1. Nonetheless, it is worth mentioning that the *sun vs shade* and its interaction with *land vs water* were both present on all seven "best" models, which account for a combined Akaike weight of 0.999, highlighting the importance of these factors. Additionally, *average (mean) air temperature* and its interaction with the *land vs water* variable were also present on all seven best models (Table S4.1). However, other parameters such as *age, sex and weather,* only appeared in a subset of the final models and were of lesser importance for explaining dorsal temperature.

Referring once again to the OLS regression between subcutaneous dorsal temperature and cloacal temperature in Experiment 2, and as shown in Fig. 4.7A, it can also be seen that the slope of the overall pattern deviated positively from isometry ($y = x$ relationship). Contrary to the pattern observed in Experiment 1, the fitted line has a slope larger than 1 (slope = 1.220, 95% CI's = 1.087 and 1.352; Table 4.2) and an intercept below zero (y-intercept = -7.861, 95% CI's = -11.774 and -3.947; Table 4.2). Furthermore, the determination coefficient for this regression (R^2 = 0.649, n = 180) was also higher than that reported for Experiment 1, yet of similar magnitude to those obtained for the OLS regressions of most other body part temperatures (regressed against T_c) tested in experiment 3 (Table 4.2).

On that note, such OLS regressions between the temperatures of several body parts tested in experiment 3, against cloacal temperature, all rendered lines with slopes smaller than 1 and intercepts considerably larger than 0 (Table 4.2). Additionally most determination coefficients, with the exception of subcutaneous neck temperature's (R^2 = 0.437, n = 1680; i.e. the lowest R^2 of all experiments), were moderate (R^2 range: 0.618-0.630) and comparable to that of Experiment 2 against subcutaneous dorsal temperature. Nevertheless, when considering stomach (instead of cloacal) temperature as the independent variable (Fig. 4.8), all determination coefficients increased considerably (R^2 range: 0.630 - 0.890) including that of the subcutaneous neck temperature (R^2 = 0.686, n = 1547), which was previously the lowest by a large margin. This left the regression between cloacal and stomach temperatures as the weakest (R^2 = 0.630, n = 908) and the one between axilla and stomach temperature as the strongest (R^2 = 0.890, n = 1547) relationships (Table 4.2).

Formula (y ~ x)	R2	Slope	Slope CI's	Intercept	Intercept CI's	P-perm	N
Eye_{IR} ~ Cloaca	0.455	0.769	0.682 0.856	4.207	1.654 6.760	0.001	363
$Dorsum_{subc.}$ ~ Cloaca	0.649	1.220	1.087 1.352	-7.861	-11.774 -3.947	0.001	180
$Neck_{external}$ ~ Cloaca	0.618	0.810	0.773 0.847	5.179	4.141 6.218	0.001	1156
$Neck_{subc.}$ ~ Cloaca	0.437	0.605	0.572 0.638	11.220	10.302 12.138	0.001	1680
$Axilla_{subc.}$ ~ Cloaca	0.622	0.724	0.693 0.754	7.857	7.005 8.708	0.001	1294
Stomach ~ Cloaca	0.630	0.740	0.703 0.777	7.434	6.410 8.457	0.001	908
$Neck_{external}$ ~ Stomach	**0.790**	1.004	0.972 1.036	-0.786	-1.706 0.134	0.001	998
$Neck_{subc.}$ ~ Stomach	0.686	0.984	0.951 1.018	0.154	-0.786 1.094	0.001	1547
$Axilla_{subc.}$ ~ Stomach	**0.890**	0.956	0.939 0.973	0.912	0.438 1.386	0.001	1547
Cloaca ~ Stomach	0.630	0.852	0.809 0.894	3.884	2.696 5.073	0.001	908
$Neck_{external}$ ~ $Neck_{subc.}$	**0.886**	1.067	1.047 1.087	-2.265	-2.839 -1.691	0.001	1384

Table 4.2: Results of the Ordinary Least Squares (OLS) regressions between the Temperature of body part$_y$ ~ Temperature of body part$_x$ .The three R^2 are shown in bold, demonstrating the best fitting models and hence to stronger y ~ x relationships. Underlined are the slopes that are not significantly different from 1, and intercepts not significantly different from zero, thus representing direct y = x relationships.

Furthermore, another pattern emerged whereby the regression lines obtained were much closer to the simple $y = x$ relationship (Fig. 4.8). In fact, for the OLS regressions of neck external (slope = 1.004; slope 95% CI's: 0.942 and 1.036; y-intercept = -0.786; y-intercept 95% CI's: -1.706 and 0.134) and neck subcutaneous (slope = 0.984; slope 95% CI's: 0.951 and 1.018; y-intercept = 0.154; y-intercept 95% CI's: -0.786 and 0.154) temperatures (both against stomach temperature), the slopes and intercepts did not significantly differ from 1 and 0, respectively (Table 4.2). Here, it is relevant to note that the aforementioned external neck temperature was also the regression showing the second highest determination coefficient (R^2 = 0.790, n = 998) of all body parts. The body

part showing the highest R-squared - the axilla (R^2 = 0.890) - had a slope very close to 1 (slope = 0.956; Table 4.2), albeit statistically significantly from it (slope 95% CI's: 0.939 and 0.973), the pattern obtained was still close to isometry (Fig. 4.8). This would also allow for a direct inference of internal body temperature albeit, in the axilla, still needing the systematic correction for the intercept (y-intercept = 0.912; y-intercept 95% CI's: 0.438 and 1.386).

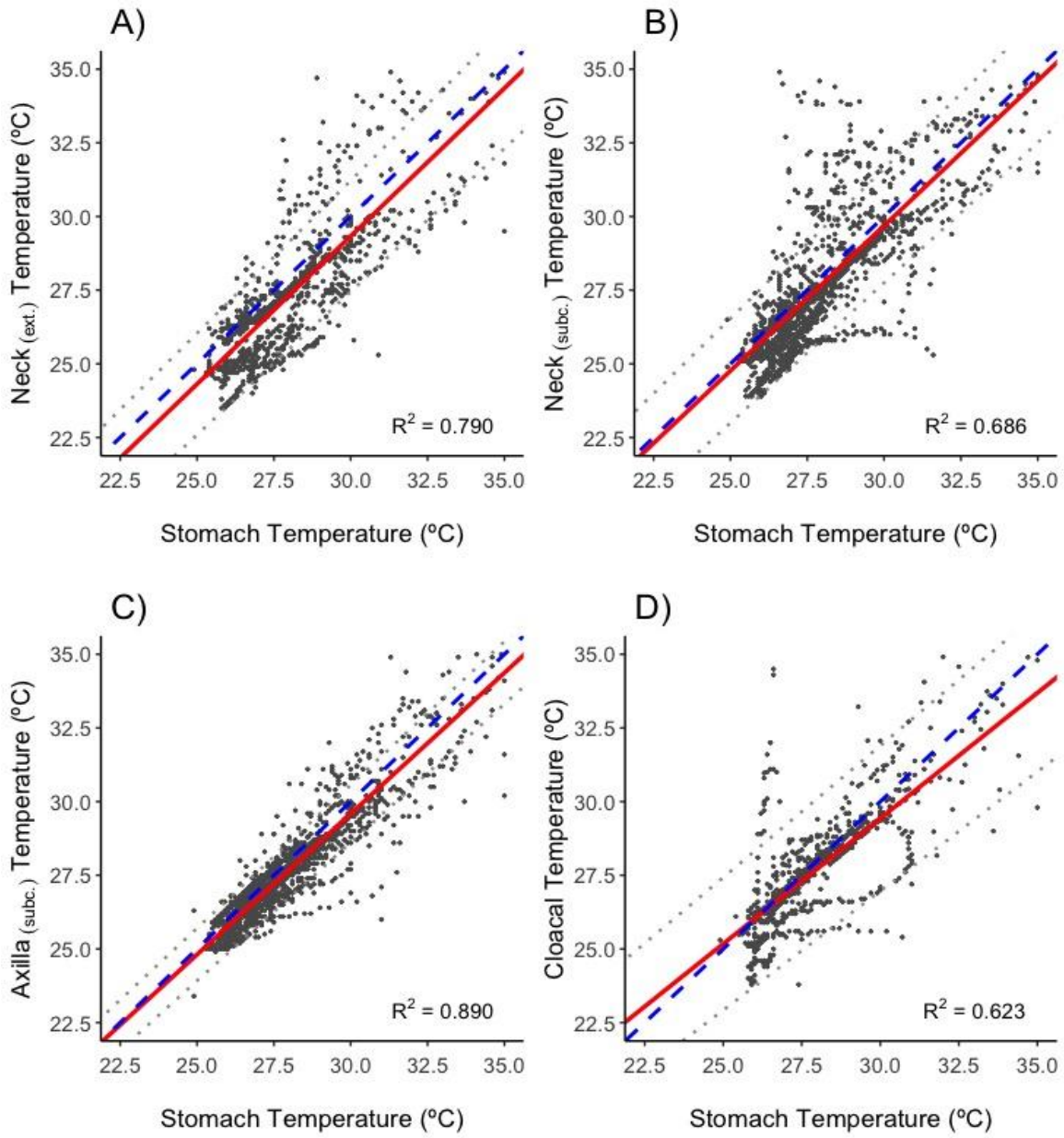

Fig. 4.8: *Plots for the ordinary least squares regression lines (red) of external neck temperature (A), subcutaneous neck temperature (B), axilla (subcutaneous) temperature (C), and cloacal temperature, against stomach temperature. 95% CI's given by the grey dotted lines and goodness-of-fit of each regression given by the respective R2. Dashed blue line representing isometric ($y = x$) relationship.*

An additional OLS regression between the neck's external and subcutaneous temperatures (Table 4.2) explored the importance of physical proximity between measuring locations and resulted in a high determination coefficient (R^2 = 0.886, n = 1384). Furthermore, the slope differed from 1 (slope = 1.067; slope 95% CI's: 1.047 and 1.087), albeit only slightly, while the intercept was considerably below 0 (y-intercept = -2.265 y-intercept 95% CI's: -2.839 and -1.691).

Further exploring the temporal pattern of temperatures of the different body parts compared to that of the stomach (Fig. 4.9), by plotting the difference in temperature between body part x and stomachal temperature ($\partial T_{(\text{body part } x) \text{ - stomach}}$) showed that on average this difference oscillated around zero (± ~1ºC). Nonetheless, the variance around the average curve varied depending on the body part and especially depending on the time of day. Such variance was lowest for $\partial T_{\text{axilla-stomach}}$. Additionally, in all cases, the variance was much larger from late morning to late afternoon (Fig. 4.9). As reported for the previous experiments, this could be indicative of other underlying variables affecting these relationships. However, due to the inherent experimental design of Experiment 3, no data was collected on some of the previously mentioned variables that could underlie this pattern (e.g. *sun vs shade*, *land vs water*).

Finally, testing which factors affected the temperature of the two body parts that provided the strongest relationships against stomachal temperature (i.e. axilla and neck external) resulted in single and similar minimal LME models for each body part (Table S4.1). As reported for the *dorsum, age, sex* and *mean air temperature* all were present in the minimal models. Additionally, *water temperature* was also present in both models. Nevertheless, the Akaike weights of these models (axilla = 0.724; neck external = 0.677) were considerably lower than that of the sum of all seven final "best" models reported for the dorsal temperature (∑ Akaike weights = 0.999), thus hinting that indeed other important variables were missing from the starting models fitted for the axilla and external neck temperatures.

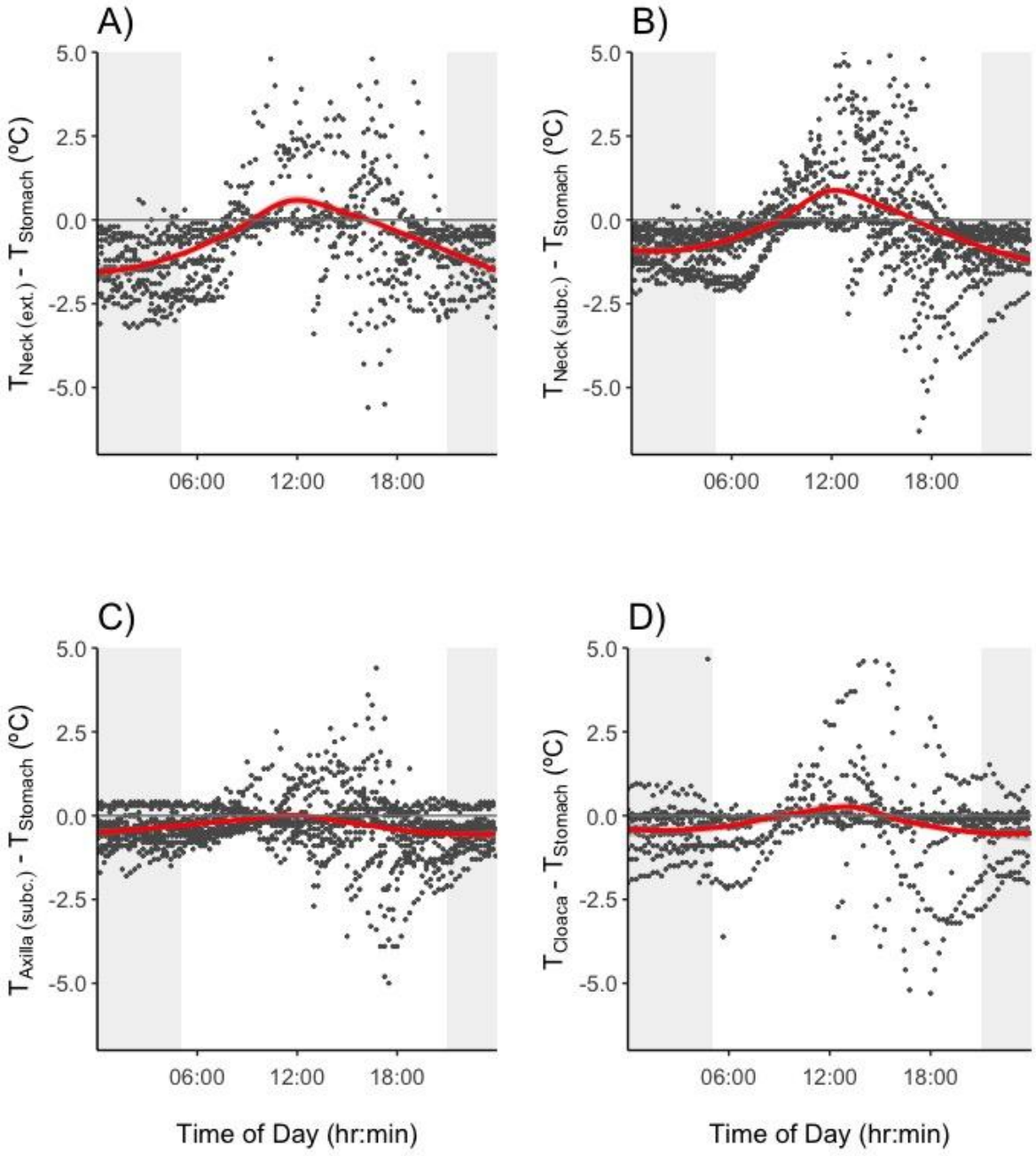

Fig. 4.9: *Plots demonstrating the difference between body parts' temperatures and internal body temperature (given by stomach temperature) across the time of the day. Difference calculated as Temperature of body partx - Temperaturestomach where body partx is A) Neck (external); B) Neck (subcutaneous); C) Axilla; and D) Cloaca. Night and daytime are represented by grey and white backgrounds on the plots, respectively.*

4.5. Discussion

This study provided robust data for a quantitative investigation of thermoregulation in *Crocodylus moreletii* by comparing temperatures of various body parts and methods of temperature measurement, assessing their accuracy, reliability, and practical applicability. By employing cloacal probes, stomach loggers, subcutaneous implants, external sensors, and infrared thermography (IRT), the results provide critical insights into crocodilian thermal physiology and the methodological challenges inherent to this field. These findings not only build upon prior research but also open new perspectives on the existence of temperature gradients and regional heterothermy in crocodilians, while highlighting the applicability of emerging technologies (e.g. integrative tags/ biologging solutions) in crocodilian thermal ecology in the interplay between terrestrial and aquatic environments. Ultimately, this study's main results can be outlined in several sub-topics, some methodological while others ecophysiological, as discussed hereafter.

4.5.1 Internal Temperature Benchmarks: Stomach vs Cloacal Temperatures

The results obtained demonstrated that stomach temperature provided more stable measures and stronger correlations than cloacal temperature, when comparing it with temperatures recorded at other body parts. The latter included subcutaneous and external measurements, across a wide range of environmental conditions (e.g. sun vs shade, land vs water). This hints that gastric temperature, when available, may be the best proxy for internal body temperature than the more widely used counterpart, the cloacal temperature.

Nevertheless, stomach's thermal stability is not surprising as its more central position in the body's core will inevitably buffer some of the thermal variability experienced by the animal's periphery resulting in well-known surface-to-core thermal gradients (Porter & Gates, 1969; Robertston and Smith, 1981; O'Connor, 1999). The effect is further magnified with increasing body sizes (Bartholomew & Tucker, 1964), such as those attained by crocodilians, which will result in larger distances between core and periphery leading to longer times until thermal equilibration between these two areas. Indeed, the same reasoning may justify the poorer performance of cloacal temperature as a proxy of internal Tb in crocodiles as, with increasing size, the cloaca also becomes increasingly distanced from the animal's centre. Its peripheric nature may lead to cloacal temperatures being more variable and often more profoundly influenced by ambient conditions such as water immersion or ground contact, as observed here. Furthermore,

the cloaca's close proximity to the highly-muscular tail also makes it susceptible to heat generated as a by-product of this body part's muscle activity (Smith, 1979), resulting in a localised temperature increase that is not representative of the overall internal state (i.e. different from mean Tb). Overall, this variability highlights a limitation of cloacal probes as a reference for internal temperature in large, semi-aquatic species like crocodiles.

These findings suggest that stomach temperature loggers may provide a more reliable baseline for understanding the relationship between internal and external body temperatures and for modelling thermal physiology in crocodilians. However, it is important to point out that stomach temperatures measured in this study were obtained from animals assumed to be in a post-absorptive state (given the farm's feeding schedule). Unlike when in an absorptive state, during which a higher perfusion (and hence delivery of heat) of the digestive organs can be expected (Axelsson et al, 1991; Farmer et al, 2008), this stage should be free of confounding effects of digestion. However, one cannot directly assume that the same patterns of heat distribution (and hence relationship between the temperature of the stomach and that of other body parts) would be observed in absorptive or starving animals (but see Brien et al, 2012). Hence, further investigation is warranted, to compare both cloacal and stomach (at different stages of digestion) temperatures to a third internal temperature benchmark: the intraperitoneal temperature, as used in several studies (e.g. Diefenbach, 1975; Seebacher, 1999; Bassetti et al, 2014). The latter was not tested here due to the considerably higher invasiveness of the necessary logger implantation procedure (see, for example, Bassetti et al, 2014), which is in fact a major drawback for the more ubiquitous use of this measure.

Nevertheless, although we hereby stand by the recommendation for further exploring the use of gastric temperature loggers to measure internal body temperature, throughout this study we have come across several practical considerations (beyond the technical characteristics of the logger, such as: non-toxic acid-resilient coating, appropriate battery life, etc.) that must be addressed for obtaining the best yield from such tools. Firstly, the size and shape must be adequate to simulate a gastrolith. This is particularly relevant in order to maximize retention times in the stomach (i.e. must be large enough to remain in the stomach for a prolonged period of time) and to prevent injury (i.e. if not smooth) or intestinal occlusion (i.e. if too big to pass naturally). This would mean that different sized-loggers (or, at least, logger-casings) would be required for differently sized animals. Further research is needed to determine the exact logger shape and size according to

the body size, as information is currently scarce (see Webb et al, 1982; and Grigg & Kirshner, 2015). Indeed, the relatively large size of the loggers used in this study limited the size of the animals that could be tested and how the logger was deployed and recovered. In fact, this prevented the animals with SVL>50cm to be investigated while for the medium-sized animals forced the attachment of the logger to the rostrum and recovery by stomach lavage (Taylor et al, 1978), instead of allowed to be naturally passed and recovered in the faeces.

Secondly, the method of deployment should be optimised as force feeding carries a degree of stress (but see Brien et al, 2012) and is not always viable in the field. Contrastingly, while the use of loggers encapsulated in food is an option to encourage a more voluntary ingestion of the sensor (e.g. Grigg et al, 1998; Seebacher et al, 1999; and as done here for the larger animals), this may only be viable with conditioned or captive animals as challenges may present when attempting to "hand-feed" a wild animal (Grigg & Kirshner, 2015). Additionally, when in group settings, we experienced difficulty in ensuring all animals ingest one baited logger each, as different animals showed varying degrees of feeding drives and/or dominance in the pecking order.

Finally, data/logger retrieval remained the biggest hurdle since "pseudogastrolith" (gastric logger) retention times vary considerably (Seebacher et al, 1999; personal observation). Hence, in order to maximise the chances of success, stomach flushing is recommended (Brien et al, 2012). Nevertheless, this procedure may be itself stressful, impractical (especially for larger animals in the field), and still it does not guarantee complete success (e.g. if the logger has since passed to the intestine). The alternative method, as used for the larger animals in Experiment 3, is to wait for the logger to simply pass with the faeces. The latter is certainly less invasive but incurs the additional inconvenience of an unpredictable and often long waiting time. In such cases, keeping the animal in an enclosed space that can be checked regularly and where an excreted logger would be more easily found is mandatory, making the process very impractical (albeit not impossible) for field contexts.

In our case, the bright colour and buoyancy of our ad hoc floating logger certainly helped in their recovery, even in a field context. Still, we did incur some losses, even in a captive (but semi-natural) context. Future gastric loggers developed could explore the possibility of a hybrid logger/transponder system that would allow periodic remote download of the stored data or at least to precisely pin-pointing the logger's location. However, such a solution would certainly be haunted by a larger size (to accommodate higher battery capacity required for signal emission) and by the high signal attenuation of water and

biological tissues (Grigg & Kirshner, 2015; Christoe et al, 2021) and hence, further thought is required to reach a workable compromise.

4.5.2 External and Subcutaneous Proxies for Internal Temperature

This study's comparison of multiple subcutaneous and external (i.e. skin surface) temperature measures revealed a significant variability in the strength of their relationships to core temperature benchmarks. Furthermore, such strength was dependent on the body location, the core benchmark used (cloaca vs stomach, as discussed above) as well as on external and environmental variables. As mentioned previously, stomach temperature yielded stronger relationships to external and subcutaneous temperatures than its cloacal counterpart, hence the former was favoured for further comparisons and discussion purposes. With regards to the body locations measured, the axillary region (measured subcutaneously under the anterior legs) and the neck region demonstrated the strongest correlations with both stomach and cloacal temperatures. Still, as mentioned in the results, the relationship between axilla and stomach temperatures differed very slightly (but statistically significantly) from an isometric relationship. Nevertheless the larger differences between the axilla OLS line and the $y = x$ line only become more apparent in the higher part of the plotted thermal range (temperature > ~33ºC; Fig 4.8C) where the animal is likely close or even past its probable thermal preference (Johnson, 1974; Brien et al, 2012) anyway. Hence, we can still claim that these sites consistently provided robust temperature estimates across the different environmental and behavioural contexts experienced by the animals throughout Experiment 3.

The reliability of the axillary region, given by the high determination coefficient obtained, likely stems from its proximity to large blood vessels carrying blood to and from the animal's thorax and head. This area is perfused by the axillary and thoracic arteries as well as by the brachial and subclavian arteries, which branch out from common carotid artery or the *collateralis colli* artery, only to be later drained mostly by the jugular veins (summarised in Grigg & Kirshner, 2015). These are major vessels responsible for the cephalic blood supply, and hence it makes sense that the temperature of the blood transported is tightly regulated due to its close proximity to the brain (but see discussion on IRT eye temperature). Furthermore, the axillary region has minimal insulation by subcutaneous fat, allowing easier access to internal thermal state. Additionally, its lateral and inherently sheltered (by the adjacent leg) position means that it will not be as

exposed to direct sunlight as other areas such as the dorsum. Hence, it will likely experience less temperature extremes caused by basking, or tightly controlled perfusion (often used to maximise thermal exchange efficiency of thermoregulatory surfaces), thus overall deviating less from core temperature values (Smith et al, 1978). Finally, the lack of osteoderms in this region, which incidentally also facilitates implantation of the logger, may also result in a less tightly regulated subcutaneous blood flow thus allowing the area to more closely follow the temperature patterns experienced by the adjacent thorax.

Regarding the neck region, however, even though both measures (external and subcutaneous) were highly correlated, a substantial difference was retrieved between the reliability of neck temperature measured subcutaneously (i.e. below a nuchal scute) or externally (i.e. skin surface temperature above a post-occipital scute) for inferring internal Tb. Interestingly, external neck temperature substantially outperformed its subcutaneous counterparts as a proxy for stomach or cloacal temperature (Table 4.2). The observed pattern likely results from the benefits of having highly vascularized osteoderms in the neck, which are known to facilitate heat exchange (Clarac et al, 2018; Clarac & Quilhac, 2019) and thus maintain close alignment with core temperatures. These findings confirm prior research emphasizing the role of vascularized dermal structures in crocodilian thermoregulation (Farlow et al., 2010; Clarac & Quilhac, 2019) as well as significant differences between subdermal and skin-surface heat flow rates (Robertson & Smith, 1979). Finally, the accessibility of both regions, as well as the negligible invasiveness associated with measuring external neck temperature, makes these locations practical candidates for sensor attachment in future studies.

In contrast, subcutaneous measurements of dorsal scute exhibited a weaker correlation with internal temperatures (although, in Experiment 2, only cloacal temperature was taken as the internal reference). This could hint at some insulating property of the scutes which may shelter the tissues immediately below. Alternatively, it could also result from the high thermal variance experienced by the dorsum – the main surface of radiant heat acquisition (Smith, 1975). The second was further confirmed by the mixed models which showed that several external factors (namely whether the animal was *on land or in water*, and *in the shadow or in the sun*) were important predictors of dorsum temperature. Furthermore, in the dorsal osteoderms blood circulation is enhanced in the surface of the scute, as opposed to the base (Clarac et al, 2018), in order to maximise the efficiency of thermal exchanges with the environment (Clarac & Quilhac, 2019). This begs the question whether dorsal skin-surface temperature would outperform its subcutaneous counterpart as a proxy of internal Tb, similarly to what was observed in the neck.

Ultimately, these results highlight the importance of selecting measurement sites that are not only anatomically relevant but also minimally influenced by external environmental conditions. Still, such environmental variables should, whenever possible, be accounted for as this could provide mechanistic estimates of internal body temperature with an even greater accuracy (Seebacher et al. 1999). Thus, a better understanding of the effect of environmental and behavioural variables on surface-to-core temperature gradients and patterns of regional heterothermy, is of paramount importance for a more robust deployment of external/superficial temperature loggers as proxies of internal state.

4.5.3 Infrared Thermography & Eye Temperature: Limitations as a Proxy for Core Temperature

The use of IRT to measure eye temperature in *C. moreletii* revealed weak correlations with cloacal temperatures, suggesting that ocular surface temperature may not be a reliable proxy for core temperature in crocodilians. This result contrasts with findings in smaller reptiles where IRT-measured [eye] temperatures are the most aligned with internal temperatures (Barroso et al., 2016 & 2020). Several physiological and anatomical factors likely contribute to this discrepancy.

First, the cephalic cardiovascular system in crocodilians plays a critical role in thermoregulation, with specialised vascular organisation working in tandem with an array of vascular shunts and sinuses (Porter et al, 2016) to enable an efficient localised heat exchange process (Clarac & Quilhac, 2019). These adaptations allow crocodilians to regulate head temperature somewhat independently of core temperature, particularly during basking or high-heat exposure scenarios (Johnson, 1974; Spotila et al, 1977). Nonetheless, such tight physiological control of head temperatures is also found in many other reptiles (e.g. Tattersall et al, 2006; Sannolo et al, 2014), including those where the IRT-measured eye temperatures strongly correlated with cloacal measures (Barroso et al, 2016). Hence, this alone does not entirely explain the low correlation obtained.

However, contrasting the vascular organisation of the crocodilian cephalic vascular system (Porter et al, 2016) to that of lacertid (Bruner, 1907) or iguanid (Porter & Witmer, 2015) lizards - cautiously, given the large phylogenetic separation between crocodilians and squamates (Irisarri et al, 2017) - reveals important differences. For instance, while for the lizards a large venous sinus – storing blood coming from the animal's core – is found immediately behind the eye (i.e. the sinus orbitalis), an analogous structure is not present in the crocodilians. Instead, the eye is perfused by several arteries which

eventually drain blood cooled in the orbital region through the orbital vein and into the cavernous sinus (Porter et al, 2016). This blood storage is located much more internally in the head, within the pituitary fossa (Porter et al, 2016), than the aforementioned sinus orbitalis of lizards, making its temperature unmeasurable through IRT measures of the eye. It does, however, influence the temperature of the pituitary gland as well as of the hypothalamus (Porter et al, 2016) – i.e. the thermoregulatory centre in the brain – and hence may indeed have an important thermoregulatory function.

Second, the large body size of crocodilians amplifies the effects of localised thermal inertia, creating significant gradients between surface and core temperatures and between body parts (i.e. regional heterothermy). This effect may be particularly pronounced in the head, where localised cooling or heating can occur rapidly due to the blood flow dynamics discussed prior and as a consequence of the head's smaller thermal inertia (compared to the abdomen and tail). This, along with head-specific thermoregulatory strategies such as gaping, panting and evaporative heat loss from the eyes and mouth, may to some degree, decouple the temperatures experienced in the head (and hence, the eye) from that of the torso.

Thus, while IRT remains a valuable tool for non-invasive monitoring of surface temperature patterns as well as cutaneous blood flow (Clarac & Quilhac, 2019), this study demonstrates this tool's limitations for estimating core temperature from eye temperatures in crocodilians. Hence, future applications of IRT in crocodilian physiology may focus on assessing broader thermal patterns and identifying regions where surface temperature might reliably reflect internal thermal states – for instance, the post-occipital and nuchal scutes, as discussed earlier.

4.5.4 Challenges Encountered and Future Perspectives: Stepping Towards Integrative Multi-Sensor Biologging Solutions

This study faced several methodological challenges, which must be considered when planning future deployment of such tools. Firstly, with regards to the use of thermal cameras, besides the fact that IRT-measured eye temperature performed poorly at inferring internal temperature, the biggest challenge was to be in close proximity to the area of interest in the animal. This meant that the animal had to be caught and restrained for such measures, thus causing comparable levels of stress (Langkilde & Shine, 2006) to that of more well-established methods such as measuring cloacal temperature via contact thermometry. Alternatives do exist where higher-end thermal cameras present

the option of fitting longer range telephoto lenses, but such solutions will likely be prohibitively expensive. Furthermore, acquiring the thermal photo of the desired location (e.g. the eye) of an animal, which must be above water, and from a large distance, may be practically difficult and will inherit further issues associated with attenuation from the environment and pixel size effects, both of which may compromise the accuracy of the reading (Faye et al, 2016). Hence, IRT may be more useful within a more controlled setting such as for laboratory or common-garden experiments, where the animals are either restrained or at least confined to a smaller space and hence more easily accessible.

With regards to the probes used in Experiments 1 through 3 to measure cloacal temperature, three different types were used: short and rigid thermocouple probe, long and flexible thermocouple probe, and NTC thermistor probe attached to a logger fitted to a harness on the back legs of the animal. The first two options were operated via a hand-held thermometer, while the last option autonomously recorded temperatures at a programmable rate (and had the option to transmit live temperature readings via Bluetooth). Both of the last two options entailed securing a flexible, thin wire inside the cloaca. This was achieved via a combination of non-toxic cyanoacrylate glue, medical adhesive and duct tape. Such attachment system did, in most cases, suffice to maintain the trailing end of the probes in place. However, the flexible nature of the probes themselves meant that the tip would often fall (or bend) out of the cloaca, needing regular re-insertion, which involved disturbance to the animal. Still, it was a better compromise than the short, rigid option which required full capture and restraint of the animal for each measurement. Finally, we also experienced issues with the harness system used to secure the logging unit of the NTC thermistor probe. Here, although the harness securely held the logger in place, larger animals were able to access the logger unit and bite it, effectively destroying it. Ultimately, whichever the contact thermometry option chosen, there was always a component of invasiveness associated with the measurement itself or the logger/probe attachment, hence, selecting the best option should be addressed on a case-by-case basis, depending on the requirements of the study.

For the smaller loggers, deployment and retrieval of stomach loggers was also problematic, especially within a natural setting, for the reasons discussed earlier. Contrastingly, for subcutaneous sensors the major set-back was the surgical procedure to implant (and retrieve) the sensor, which required a degree of veterinary resources and expertise that may be prohibitive in some contexts. Nevertheless, this prerequisite will be heavily dependent on the size and type of sensor to be implanted (e.g. PIT tags can

be easily injected with no need for surgical equipment, skill or analgesia) and the location in question. Retaining the subcutaneous logger in place may also present some challenges. In our case, particularly for Experiment 2 where we only used surgical glue to close the incision, some sensors dislodged during the experiment. This issue was resolved for Experiment 3 by suturing the incision shut with 2-3 sutures. In either of these experiments, there were no signs of the sensors migrating within the body or being ejected, as sometimes reported in other studies (Whitaker & Srinivasan, 2018; Hocutt, 2022). Nonetheless, most of the tested animals were only tagged for very short periods of time (1-7 days), which may be too short for any noticeable changes in sensor position. Hence, further investigation is warranted to address this potential issue.

With regards to the floating logger, which was deployed in Experiment 3 to measure temperatures of the skin surface, stomach and the cloaca, although we ultimately do consider the design a success for our desired outcome (i.e. easily deployed and self-detaching logger for short periods of intense sampling in animals in a confined space), several issues hinder a more general, long-term use. Firstly, the method of attachment to external surfaces (through the use of non-toxic surgical glue), albeit facilitating an easy and harmless retrieval, meant that the logger was only secured for two to three days maximum. This timeframe would certainly be insufficient for long-term studies and hence alternative methods of attachment and self-release should be considered (see Franklin et al 2009; and Brien et al, 2010). Secondly, after detachment the logger successfully floated, however, its size made it difficult to locate in more complex/natural environments (especially when dislodged on land), even with its bright-yellow colour purposely devised to make the logger more conspicuous. Future versions of small, self-detachable sensors could consider brighter or reflective (even UV reflective) non-toxic paints that better contrast with the expected surrounding in order to facilitate search and retrieval after the logger detaches. Finally, unless the animal is under constant monitoring, it was impossible to pinpoint the exact time in which the logger detached, hence data had to be extensively trimmed in order to infer a conservative estimate of when the temperature measures are no longer of the subject of interest. Again, this could be addressed with better attachments that ensure a more consistent and predictable self-release time (e.g. reviewed in Franklin et al, 2009).

Furthermore, the results of the LME models fitted in this study demonstrated the effect of external factors, such as being in water or on land, or in the sun vs in the shade, on the inference of body temperatures. This highlights the importance of incorporating diverse environmental and behavioural contexts when estimating internal body

temperatures. Thus, in addition to thermal sensor(s), future tools should strive to integrate complementary sensors, such as accelerometers, magnetometers, gyroscopes, wet-dry sensors, light intensity meters, etc, following a general trend towards developing multi-sensor biologging systems (Franklin et al, 2009). These sensor arrays could provide valuable contextual data on activity states, body postures, submersion patterns, and habitat use (as reviewed in Franklin et al, 2009). For example, sun/shade sensors could capture thermal gradients within microhabitats as well as determine when an animal is basking, while wet/dry sensors could elucidate time spent in and out of water. Altogether, these variables, combined with surface/subcutaneous temperature measurements (of post-occipital or nuchal scutes, for example), could be used to mechanistically infer core body temperature data, as well as provide a holistic view of crocodilian behaviour, ecology and energetics. Similar systems are increasingly used in both aquatic and terrestrial fauna (Brown et al, 2013) but have been slow to percolate into the field of reptile ecophysiology (but see Franklin et al, 2009).

Nevertheless, the need for proper calibration of all these measures should be again emphasised. Standardized calibration protocols, tailored to specific sensors and environmental conditions, are essential for ensuring accuracy and comparability across studies. Future efforts should prioritise the development of robust calibration frameworks, informed by both experimental data and field observations. Still, multi-sensor approaches inevitably present challenges, including increased tag complexity and size (with increasing number of sensors), potential impacts on animal behaviour, and the logistical demands of data integration and analysis (briefly reviewed in Brown et al, 2013). Ensuring that biologging devices are lightweight, durable, and minimally intrusive will be critical for their success. Additionally, interdisciplinary collaboration between ecologists, physiologists, veterinarians, managers and engineers will be essential for developing innovative solutions that balance data richness with animal welfare considerations.

Ultimately this study contributes with a set of initial steps towards a crocodilian-oriented integrative tag, as pioneered by the studies reviewed in Franklin et al (2009). Here we highlight the utility of axillary and external neck temperatures as reliable proxies for core temperature, suggesting that sensors targeting these sites should be prioritised in biologging designs that wish to explore crocodilian thermal physiology.

4.6. Conclusion

This study provides important insights into crocodilian thermal physiology by evaluating the performance of various temperature measurement methods in *C. moreletii*. The study highlights the reliability of stomach temperature as an internal temperature reference, as well as the utility of axillary and external neck temperatures as proxies of core Tb, while uncovering the limitations of IRT for estimating core thermal state. The results obtained emphasise the value of integrating body temperature data with complementary physiological, behavioural and environmental variables, offering a comprehensive framework for understanding crocodilian ecology.

As climate change alters thermal landscapes, integrative biologging approaches will be essential for predicting crocodilian responses and informing conservation strategies. Future research should prioritise the development of robust, multi-sensor biologging systems that incorporate key variables such as activity state, sun exposure, and water immersion, advancing our understanding of crocodilian thermoregulation and adaptation.

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4.9. Supplementary Materials

Table S 4.1: *Best (∆AICc < 2) models resulting from model selection of linear mixed effects (LME) models for the response variables ("y"): A) dorsum temperature (Experiment 2); B) axilla temperature (Experiment 3); and C) neck (external) temperature (Experiment 3). Explanatory variables (fixed terms) present in each model given by "+" and absence indicated by blank space. "NA" represents fixed terms that were not tested in the full/initial model for that response variable. Only variables that were relevant for at least one of the best models - from either A), B) or C) - are shown here. Given the repeated measures structure of the data, all models included animal ID as a random factor. Full structure of the full/initial models (for each response variable "y") available in the published dataset and analysis script as well as described in the Statistical Analysis section of the article.*

y	Rank	(Intercept)	Age	Land vs Water	Sex	Sun vs Shade	Avg Air Temperature	Water Temperature	Weather	Land vs Water : Sun vs Shade	Land vs Water : Avg Air Temperature	df	logLik	AICc	ΔAICc	Weight
	1	19.57		+	+	+	+	NA	+	+	+	10	-269.134	559.601	0.00	0.222
	2	19.40	+	+	+	+	+	NA	+	+	+	11	-268.230	560.069	0.47	0.176
	3	19.57		+		+	+	NA	+	+	+	9	-270.568	560.221	0.62	0.163
A	4	19.53		+	+	+	+	NA		+	+	9	-270.809	560.703	1.10	0.128
	5	19.47	+	+		+	+	NA	+	+	+	10	-269.785	560.903	1.30	0.116
	6	19.40	+	+	+	+	+	NA		+	+	10	-269.939	561.210	1.61	0.099
	7	19.50		+		+	+	NA		+	+	8	-272.215	561.293	1.69	0.095
B	1	-13.25	+	NA	+	NA	+	+	NA	NA	NA	7	-741.853	1498.000	0.00	0.724
C	1	-17.26	+	NA	+	NA	+	+	NA	NA	NA	7	-668.324	1351.000	0.00	0.677

5. Chapter 5 - Husbandry guidelines for the safe brumation of two lacertid lizard species (*Iberolacerta monticola* and *Podarcis lusitanicus*) in laboratory conditions

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This chapter is based on content that has since been published as a protocol:

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5.1. Abstract

Lacertid lizards are often used for a range of laboratory studies in reptile behaviour, physiology, ecology etc. However, there is a distinct lack of primary information regarding the husbandry of these animals under captive, laboratory conditions. This lack of information is even more apparent when looking for standard husbandry best practices for the brumation of small lizards in a captive setting. Hence, when faced with the need to overwinter captive populations of two lacertid lizard species from a montane environment (*Iberolacerta monticola* and *Podarcis lusitanicus*), the need arose to establish a conservative protocol that would allow the animals to safely go through their seasonal activity cycle (and brumate) in a controlled laboratory environment. This protocol therefore addresses that gap as it aimed to simulate a simplification of the conditions thought to be experienced by these animals while brumating. Ultimately, through this protocol, these species were successfully overwintered in a laboratory setting without any casualties (during the brumation period) and with no significant effect on the body condition of the animals. Hence, this protocol hopes to provide a baseline framework, on which other studies may capitalise, build upon or adjust, for the safe brumation of lizards in a laboratory setting.

5.2. Background

Many animals resort to some level of dormancy in order to cope with periods of scarce resources or harsh environments. Hibernation in mammals is a textbook example of such a period of inactivity and physiological latency (Mohr, Bagriantsev & Gracheva, 2020). Nonetheless, analogous inactive states exist in many other groups including reptiles, where they are termed aestivation (over Summer, often observed in xeric or tropical species - Macip-Ríos et al., 2023) or brumation (over Winter, often observed in alpine or temperate species - Nordberg & Cobb, 2017).

Importantly, winter dormancy in reptiles is not necessarily a simple “cold-induced torpor” where inactivity is merely forced by low ambient temperatures. Instead, brumation can involve coordinated and seasonally structured physiological changes, such as metabolic depression beyond simple temperature dependence and shifts in hormonal regulation and energy balance (Gangloff et al., 2016), that facilitate entering, enduring, and emerging from prolonged cold exposure (Holden et al., 2021).

In this sense, brumation is better understood as an integrated seasonal phenotype, in which temperature interacts with endogenous state and environmental cues (including photoperiod - Moss et al., 2022), rather than as a passive consequence of being cold. Classic experimental work on reptiles explicitly highlighted this separation between dormancy and metabolic regulation, and even proposed the term brumation to distinguish winter dormancy in ectotherms with physiological changes that can be partially independent of instantaneous body temperature (Mayhew, 1965).

Nonetheless, despite this general knowledge that these animals brumate, there is a remarkable absence of published primary information regarding the conditions in which these animals do so, either in a wild or captive setting. Furthermore, many such species, including some lacertid lizards, are often found in private or zoological collections, or commonly studied species in a captive/laboratory setting. Hence it is crucial to have best practice guidelines on the husbandry of such species, both for their active and inactive periods.

This knowledge gap is not merely academic: inadequate husbandry practices can have both direct welfare consequences (e.g., dehydration, poor body condition, disease) (Denommé, Bakker & Tattersall, 2025), as well as important methodological repercussions for studies that rely on behaviour and thermoregulation as response variables. Yet while there is a growing amount of evidence for implications of inadequate husbandry conditions during active periods (e.g. Denommé, Bakker, & Tattersall, 2025; Hornby & Hedley, 2024), the same is not the case for inactive stages such as brumation or aestivation (Blais et al., 2022; Jiang et al., 2023).

As widely discussed in the literature, lizard thermal ecology is strongly mediated by behaviour (Giacometti et al., 2023; Sales & Freire, 2019), hence captive conditions can distort natural thermal opportunities, activity cycles, or recovery trajectories have clear potential to bias subsequent behavioural and ecophysiological inference.

In addition, practical husbandry decisions around overwintering (e.g., when to cease feeding, when to reduce temperatures, how to manage hydration) are tightly linked to basic brumation physiology. For instance, prolonged cold exposure is associated with low metabolic throughput and altered energy regulation (Dubiner et al., 2023); consequently, ensuring that animals enter brumation without undigested food is a common-sense preventive measure, because gut stasis and obstruction can be catastrophic if animals cool while still processing food -as illustrated by documented

faecolith-associated obstruction during brumation in reptiles (Corbit, Person, & Hayes, 2013; Louth & Heatley, 2020).

Finally, for wild-caught animals intended for release, brumation protocols must also be considered within a broader ethical and biosecurity framework. Standard herpetological guidelines (Baupre et al., 2004) emphasise that release should be limited to short-term captivity, healthy individuals, isolation from other collections/exotic species, and release at the original capture site under conducive seasonal conditions. These are precisely the constraints that induced the need to overwinter the animals of this protocol.

This protocol wishes to address that knowledge gap by resorting to some information compiled (for other species) from private lacertid keeper forums, as well as on an informed attempt to simulate the most realistic recreation of the winter-time environment in the animals' native ranges, in order to suggest a replicable and adjustable methodology for the safe brumation of lizards in a laboratory setting.

5.3. Protocol Aim

This protocol was devised to brumate a large number of wild lizards temporarily maintained in captivity following behavioural and physiological experimentation in Autumn 2020. Because release during winter would have exposed animals to harsh environmental conditions, individuals were retained until Spring 2021. To avoid disrupting natural seasonal cycles, conditions conducive to brumation were devised and implemented in a laboratory setting.

Ultimately this protocol hopes to provide a baseline framework for the safe brumation of lizards in a captive state. Nonetheless, caution is advised when extrapolating this protocol towards species beyond those described here and which may have different activity/physiological cycles or be exposed to different wintertime environmental conditions. Hence, it is advised that this protocol is taken as a baseline framework rather than a prescriptive standard. Species-specific ecology and natural winter conditions should always guide any further adaptation and deployment of this methodology.

Furthermore, it is important to note that this protocol was devised under a lack of official husbandry/brumation guidelines for the species of interest and from the country where the study was conducted. Nonetheless, prior to deploying this protocol to any other

location and/or species, a thorough search for the local official guidelines and legislation applicable to the species in question must always be performed.

5.4. Animal subjects used for this protocol

The animals in question were adults of the species *Iberolacerta monticola* (12 males, 8 females) and *Podarcis lusitanicus* (12 males, 17 females; formerly *P. guadarramae lusitanicus* – Caeiro-Dias et al., 2021) collected from Serra da Estrela (district of Guarda, Portugal). At approximately 2000m and 1400m elevation, respectively, these montane populations experience persistent winter snow cover and brumate in burrows and rock crevices (Matos et al., 2011).

The high-altitude origin of these populations motivated a conservative protocol emphasising controlled thermal transitions, stable low-temperature maintenance, and monitoring of body condition (Caeiro-Dias et al., 2021), while approximating buffered overwintering microhabitats typical of alpine environments. Implementation remained compatible with ethical repatriation principles for medium-to-short-term captive husbandry of wild animals (Baupre et al., 2004).

5.5. Before Brumation

-Enclosures:

Males were kept individually in separate terraria (30Lx20Wx17H cm) and provided with a small brick for shelter and thermoregulation. Females were kept in communal enclosures (46.5Lx37.5Wx23.5H cm, one for each species) with several stacked bricks to provide shelters and thermoregulation platforms. It is important to note that these species, especially *I. monticola*, are often found congregating in communal shelters or basking spots (personal observation).

-Food and water:

Animals were provided with water ad libitum in water dishes (individually for males and communal for females) and lightly misted, on a daily basis, in order to maintain environmental humidity. Lizards were fed Tenebrio molitor larvae, dusted with a multivitamin powder, 3 times a week. At times, crickets (*Acheta domesticus*) were made available instead.

-Photoperiod:

Natural photoperiod and radiant thermal source were provided via a diffuse glass window and an array of 150w infrared basking lights, at a 2-3m distance from the terraria. Photoperiod followed the progression that the animals would experience in the wild since the lab was at a similar latitude to the animals' sites of capture. As Winter approached, the animals became increasingly less active and less interested in the food supplied. Perhaps triggered by the naturally decreasing photoperiod, temperature or other unknown intrinsic factors. To match this, within the month prior to moving the animals into the brumation chambers, the time of basking lights available was gradually decreased until a minimum period of 5 hours (always concentrated around mid-day).

-**Air Temperature Control:**

Ambient air temperature in the facility was maintained around 18ºC via an air conditioning (AC) unit.

During the pre-brumation period, air temperatures were also allowed to fluctuate more naturally along the day by turning off the AC from late afternoon until the following morning. As days got progressively cooler outside, so did the lab itself. Although, still providing an increasingly short window of sufficient warmth for the animals to feed and digest (through a combination of AC and basking lights). As the animals' physiology changed and their feeding drive decreased, the frequency of feeding was also decreased, to a point when animals were not fed at all (for a minimum of one week prior to being transferred into brumation chambers). This was to ensure that food was not consumed and left undigested during the long brumation period. Otherwise, undigested food could potentially lead to severe constipation (formation of faecoliths) or decompose causing internal infections, sepsis and, ultimately, death of the animal (Corbit, Person, & Hayes, 2013; Louth & Heatley, 2020).

-Monitoring:

Body condition of the animals was monitored through monthly weightings in a precision balance (Sartorius M-Pact AX224, Sartorius AG, Germany; precision: 0.0001g). This was done to ensure the animals started brumation with an adequate condition (i.e. were neither too thin nor in a trend of sharply decreasing weight) as well as to then evaluate the impact of brumation. To avoid disturbing the animals during the brumation process, during such time, animals were only visually inspected to assess their general condition

(see Regular Monitoring section below). Regular (weekly) weightings were only resumed post-brumation, to assess the effects of brumation on body condition and track the subsequent recovery.

5.6. Brumation Set-Up

Animals of both these species are often found in the wild aggregating in the same crevices and/or basking spots (pers. obs.). Hence, partly due to space constraints, animals were housed in communal brumation terraria.

It is important to note that all animals came from the same population (of each species) and that, when placed in brumation, no animal was showing signs of poor body condition or disease/parasitization. Where that the case, it would then be advisable to either quarantine the animal(s) and potentially abort brumation altogether, should the animal be too weak.

Nevertheless, while in the absence of space and/or equipment constraints individual housing of brumating lizards may be preferable from a monitoring, husbandry and biosecurity perspective, it is also important to consider the potential social and thermoregulatory benefits of “huddling” behaviour (Iryshkov et al., 2024; Reiserer, Schuett, & Earley, 2007; Shah et al., 2003), yet untested for these species. Hence, the choice for communal housing still stands.

-The Terraria:

Each brumation terrarium housed 8-12 animals of the same sex and species. Furthermore, it is important to reiterate that, even if from the same species, only animals from the same population should be housed together, and any animal showing signs of bad body condition or disease/being parasitized must be kept isolated or even considered to exclude from brumation.

The terraria used in the first trial deployment of this protocol were simple, transparent plastic storage boxes (dimensions: 40Lx29Wx15H cm; volume: 12 litres) with a locking mechanism for the lid. Presence of a locking lid is essential to prevent animals from escaping as some animals can be strong enough to push their way out if not properly locked (pers. obs.). The lid itself was modified by creating many evenly spaced ventilation holes across its entire surface (diameter ≤ 5mm but adjust to species size; Fig. 5.1). Taller boxes (dimensions: 40Lx29Wx25H cm; volume: 20 litres) were

subsequently used in a further study (Barroso & Sreelatha et al., in preparation) on the same species, which enable more volume and the ability to create more shelters and a vertical gradient of temperature and humidity (Fig. 5.1).

Furthermore, in order to prevent extensive direct contact between the bottom of the terraria and the metal floor of the brumation chambers, the external bottom sides of the brumation terraria were insulated with composite cork sheets. Metal (from the chambers' floor) tends to draw away heat very fast from a body and this could increase the risk of inducing hypothermia or even killing the animals by a fast cold-shock. As an additional preventive measure, wood boards were also used to lift the terraria from the metal surface (Fig. 5.3).

Fig. 5.1: Photos of the brumation terrarium set-up (12 litre version).

-The Substrate:

In an attempt to create a substrate humidity gradient, each terrarium was split in half. One side took a thin (~1cm) layer of sand mixed with vermiculite (<10% volume), and the other a 1-2 cm deep layer of "bioactive substrate" (see "Bioactive Substrate" section for details) (Fig. 5.2). A subsequent deployment of this protocol successfully recreated a more stable hydric environment by using a deeper layer of bioactive substrate (5-10cm). This provided further microhabitats for the animals to dig and brumate into, as well as better moisture retention properties (i.e. no need to spray as often, meaning less disturbance).

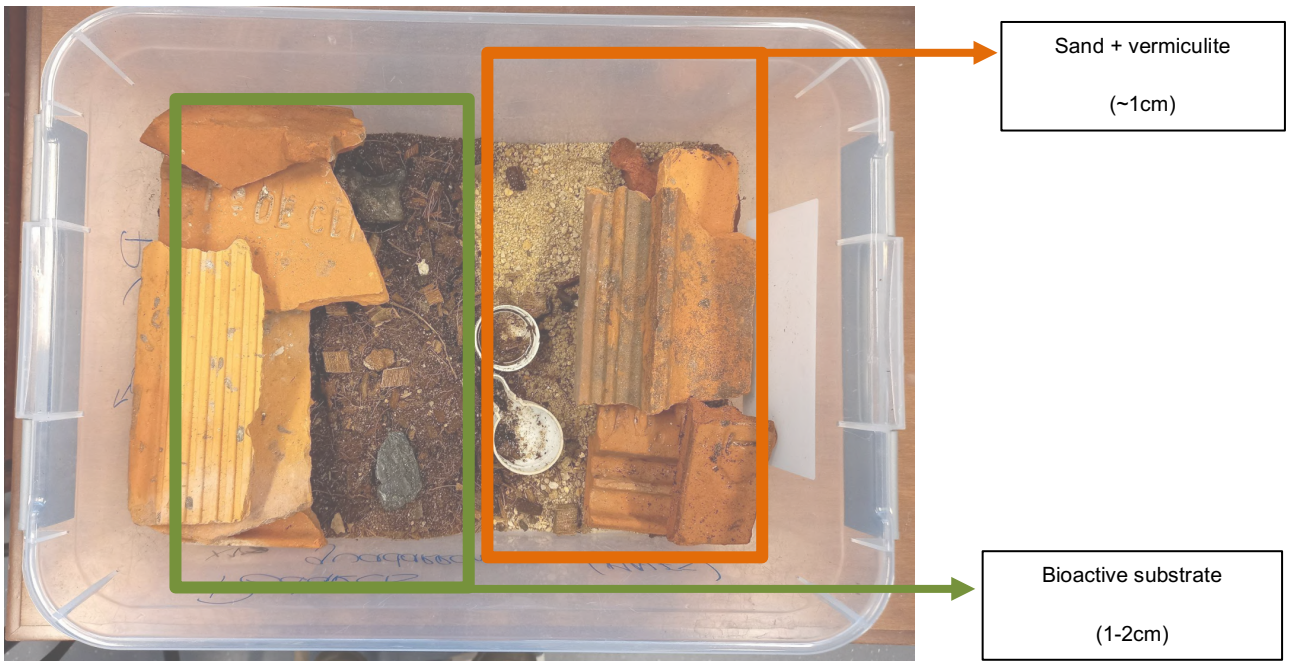

Fig. 5.2: Substrate distribution in the brumation terrarium

-The Refugia:

To create refugia, layers of pieces of bricks/tiles were carefully stacked in opposite ends of the terrarium (Fig. 5.1), leaving 1-2cm tall (species dependent) crevices between layers. Much care was taken to ensure stability of the final structure as these animals are deceptively powerful at moving even larger pieces of brick (pers. obs.). In order to facilitate access to visually inspect the animals, these pieces were not glued together, and it was really strived for mechanical stability when arranging the pieces. However, gluing the pieces together is indeed the safest option to avoid any animal being inadvertently crushed. To do so, the best options would be to use 100%/Aquarium-grade silicone, “super-glue” (cyanoacrylate glue) or careful use of expanding polyurethane foam (with no additives). These however, each have very specific curing times, but all eventually render completely safe and non-toxic solutions when fully cured, as is evident by their ubiquitous use amongst aquarium and terrarium keepers.

The stacks of bricks were created as tall as possible but also allowing for enough space for the animals to move between layers of bricks and between the top layer of the structure and the lid of the container. The latter was in fact very often used (particularly

by the *Iberolacerta monticola*). Additionally, some free space of just soil and sand was left open, in between the stacks (Fig. 5.1).

-**Water:**

A couple of water dishes were supplied in the middle of the terraria (Fig. 5.1 & Fig. 5.2). These allowed the animals to drink or soak but also contributed towards maintaining a high relative humidity inside the terraria, aided by periodic misting and by the high humidity content retained in the bioactive soil mixture.

-**Biosecurity Measures**:

Given that these are animals collected from the wild, to where they have to be returned, there is an added importance of maintaining proper biosecurity protocols that prevent potential cross-contaminations/ spread of diseases/parasites between species and/or allopatric populations. Hence why careful considerations must be taken with regards to housing arrangements (e.g. see above considerations regarding communal housing per species). Furthermore, a proper hygiene protocol is essential for preventing such cross-contaminations.

Thus, all bricks and water dishes used in the terraria were previously sterilised by soaking and washing them in solution containing bleach (household cleaning bleach ~5-10ml in 10 litres of water). These were then thoroughly rinsed and the water (an any remaining bleach) left to evaporate over the course of a couple of days.

The bricks were then further sterilised by placing them in an oven at 80ºC for a minimum of 30 minutes. The substrate systems also underwent a heat-sterilisation process (baked at 80ºC for over 1 hour). However, after such treatment, the bioactive substrate must once again be rehydrated and recolonised with native isopods and collembola (the bioactive clean-up crew; see “Bioactive Substrate” below).

-The Brumation Chambers:

With regards to the brumation chambers themselves, the ideal would be to use climatic/environmental chambers where the user has full control over the set environmental conditions (temperature, humidity, light intensity and period, etc.) (e.g. Le Galliard et al., 2021). However, these are prohibitively expensive for the scope of these, and many, studies and hence cheaper alternatives were devised for this protocol.

The initial approach, here described, resorted to the used of laboratory incubation chambers (Binder KB53, Binder Inc., USA; temperature range: -5 – 100ºC, capacity: 51l)

(Fig. 5.3 right). The limited space available in each incubator meant two of such units had to be used to house all the terraria. However, larger models do exist and would be recommended.

The incubators were set to a fan speed ≤30 (on a scale of 0 to 100) and their high precision of control of the conditions meant that no significant difference would be expected between the environment experienced inside both incubators. These have the further advantage of allowing for automatic cycles of varying temperatures to be programmed, which could be useful under certain scenarios. Nonetheless, for the purpose of this proof-of-concept, temperatures were manually set as required.

The inner, glass door of the chambers was closed but the external (metal) door was always left open to allow some photoperiod for the animals. Additionally, an array of 70W incandescent lights was placed facing the incubators from ~1.5 metre distance (Fig. 5.3 left) and programmed to be on for 5 hours in the late morning to mid-afternoon. These reinforced the photoperiod (provided by the lab's window) but also provided a short window of basking opportunities for the animals, should they choose to take advantage of it.

As an additional safety precaution, any holes larger than 1cm in diameter leading to the internal components of the incubators (e.g. access to the fan) were covered with fine netting to account for the eventuality of an animal escaping into the fan/internal compartments of the equipment.

Alternatively, a second deployment of this protocol resorted to the use of a commercial vertical refrigerator for beverages/cakes (Fig. 5.4 left). Unlike household refrigerators, these tend to have a transparent glass door which proves crucial for manipulating the photoperiod as well as for visual inspection of the terraria without the need to open the unit (which would affect its temperature). These are also a more economical option than laboratory-grade incubators and allow for a sufficient temperature control for the purpose here described - usually around the 0-10ºC range (sufficient for the species in question but may not be adequate for all species).

However, the embedded temperature control of these refrigerator units is neither as precise nor as accurate as the incubators, hence it is important not only to measure the temperatures before adding the lizards but also to monitor it through time. Furthermore, the compressor in these refrigerators does not run continuously and usually has a threshold of around +/-2ºC (depending on the model – usually explicit in the technical booklet of the product) around the temperature it's set to, around which it fluctuates in a

cyclic manner (see graph in Fig. 5.4). Hence it is very important to measure this in order to select the best setting before use, but also to closely monitor these fluctuations during the use (e.g. via temperature dataloggers).

Fig. 5.3: Array of lamps and lab window that supplied the photoperiod and basking opportunities for animals during brumation (left); Binder KB53 incubators used as brumation chambers (right)

Additionally, these refrigerators do not usually allow control of the air flow. They will have an air flow vent from which they recirculate cold air across the unit to cool it down. This vent is usually found in the top of the unit, as cold air is then expected to sink. However, this means that a vertical gradient of temperatures could arise inside the unit. To avoid this, simple, quiet USB fans (easily acquired online) (Fig. 5.4 left), were run continuously at the bottom of the unit promoting a more homogenous distribution of temperature. Nonetheless, close monitoring of temperatures along the different levels of the unit is highly recommended before and during its use.

Ultimately, even though no information was available on the exact temperature and their stability inside the natural hibernacula of these species, these fluctuating and less precise conditions where successfully used by Barroso & Sreelatha *et al.* (*in*

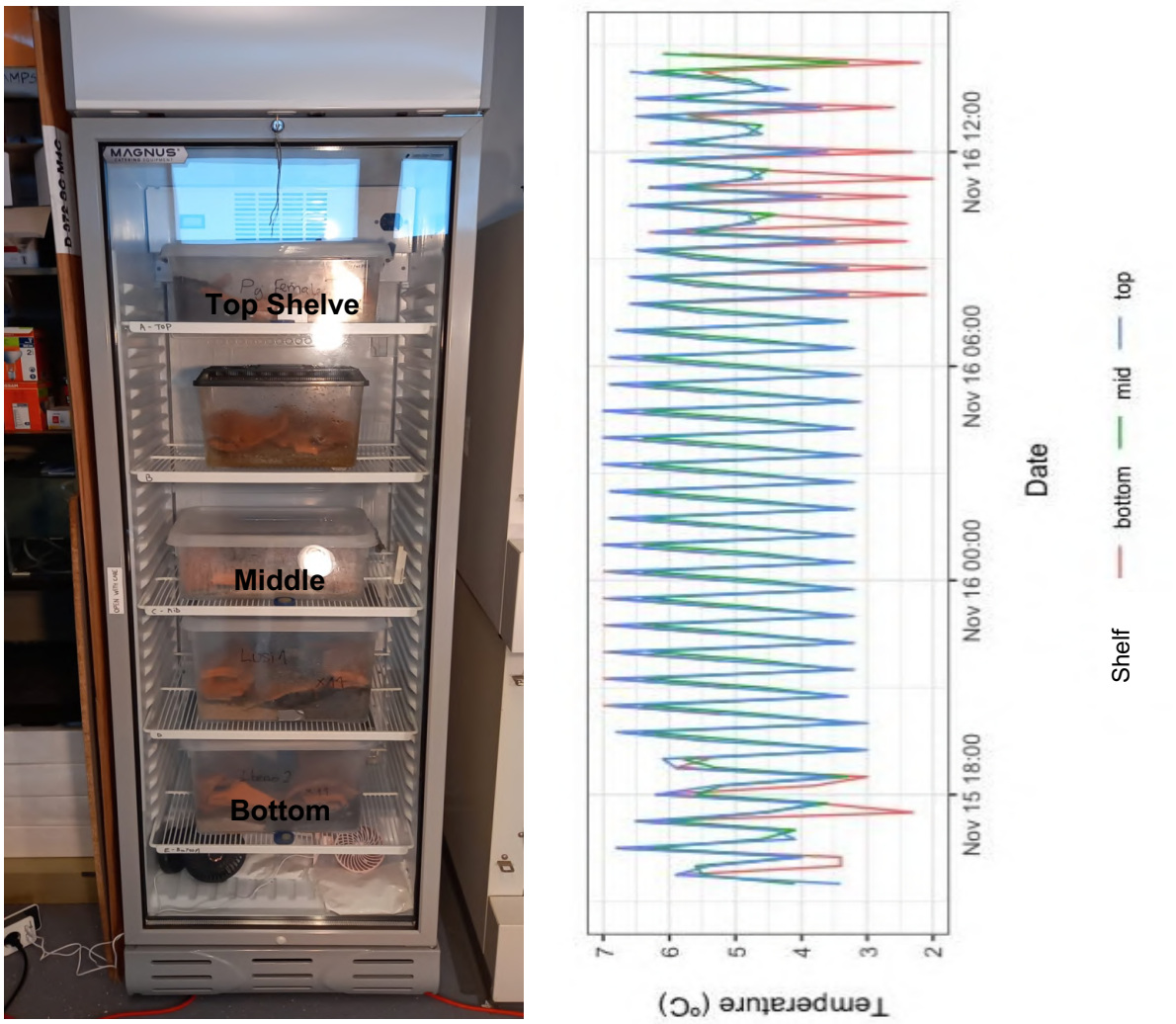

Fig. 5.4: Commercial refrigerator (left) used where fans for air movement are visible (below bottom shelve) as are the small dataloggers (small grey circle inside blue cap in top, middle and bottom shelves) used to monitor temperature profile; Temperature profile (left) of each shelve measured showing temperature fluctuations over a 1-day period.

preparation), for the same two species, in the follow-up study aforementioned. The refrigerator used for such study was the model "Magnus M&S D 372 SCM 4C" (temperature range: 0-10ºC, power: 0.35kW, Capacity: 325 litres) (Fig. 5.4 left).

5.7. During Brumation:

-The Conditions:

After being placed in the brumation chambers, the temperature was gradually decreased from 15ºC (ambient temperature) to 5ºC over a period of 2-4 weeks. A temperature of 5ºC and fan speed = 30 were the conditions left for the remainder of the brumation period. Natural photoperiod was maintained by indirect sunlight from a frosted glass window and supplemented by an array of 70W incandescent lights (Fig. 5.3 left) facing the incubators from 1.5 metre distance. The latter were programmed to be on for 5 hours in the late morning to mid-afternoon. Additionally, feeding was entirely suspended throughout the brumation period.

Throughout the entire brumation period, the incubator glass door was periodically opened and allowed to ventilate (to exchange the air inside) for a couple of minutes (3 times a week when using the [smaller volume] incubators, once a week for the larger refrigerator). At this point the lab's AC was turned off so outside temperatures did not represent a large difference to that of the incubators. Furthermore, the incubators rapidly recovered the desired temperature once the door was closed.

-Regular Monitoring:

Once a week, all the terraria were opened and visually inspected (without disturbing the animals, unless necessary). Several aspects were checked:

- Soil moisture – the bioactive soil should be moist (not wet, and still loose; Fig. 5.8). If such was not the case, then the terrarium was lightly misted. In fact, most of the times the terraria were actually too wet (from condensation), which was addressed by increasing the number of holes in the lid of the terraria as well as adding some (very few and very small <2mm diameter) holes on the sides of the terraria. Small holes in the bottom of the terraria should also be considered in order to drain excess water that may accumulate under the substrate system, but a collection system (e.g. tray) and emptying routine must be devised to prevent

water accumulating and potentially freezing when in contact with the base surface of the incubator.

- Presence of ice formation (in the incubator/refrigerator or inside the terraria) – the brumation chambers and the terraria were visually inspected for indications of ice. This should be avoided at all costs to prevent animals from freezing or suffering frostbite, as well as to avoid damage to the unit.
- The water dishes still have water and its clean (refilled/ replaced as needed with tap water, ideally rested for 24hr to evaporate any chlorine in the water).
- If there are signs of bricks being dislodged and if so double check that no animal is trapped (which never happened, in our deployments but is a risk if bricks are not glued) and re-secure the bricks in question. In the animals that are visible (i.e. we did not actively search for every animal, every time in order to avoid unnecessary disturbance to the terraria), check for general health signs such as apparent loss of mass, discharge from mouth or nose, presence of fungi/external parasites/wounds/frostbite, etc. This must be done with minimal disturbance to the animals (i.e. without touching them unless a problem was indeed suspected). Nevertheless, issues with the animals during the brumation period were rarely seen. However, had there been any such suspicion, the animal(s) in question would have been removed and placed in an individual terrarium to avoid propagating the condition. Depending on the severity of the situation, terminating brumation should be considered to allow the animal to attain body temperatures high enough to resume feeding and hence speed the recovery/healing process. This, however, should be avoided as suddenly terminating the brumation may present a big physiological shock to the animal. This visual inspection was easier for Iberolacerta monticola since they tended to lodge themselves between the top bricks and the lid of the terraria (Fig. 5.5), as opposed to the Podarcis lusitanicus which more often hid in between the layers of bricks.
- Temperature and humidity measurements from data loggers (some inside the brumation chambers but out of the terraria, others inside the terraria themselves) were downloaded and checked for any abnormalities or concerns

Fig. 5.*5:* *I. monticola* (males) in their brumation terrarium during one of the periodic checks. They were often found clumped together at the top of the terraria

-Managing Relative Humidity:

It was also not infrequent to observe large amounts of condensation happening both inside and outside of the incubators/refrigerator doors. This did not necessarily represent an issue to the brumating animals, yet it often led to the pooling of some water either inside the chambers or on the floor of the lab, potentially leading to water damage to those. This could result from maintain the brumation terraria too humid, at which point the amount of misting must be addressed.

Alternatively, it could also result from humidity in the outside air condensing in the cold surfaces of the incubators/refrigerators. To address these, it was opted to use a dehumidifier in the laboratory and an air circulation fan pointing towards the door of the chambers. These promoted the evaporation of any water condensing on these surfaces.

Issues with condensation inside the units were dealt by adjusting the amount of misting as well as by lodging a small piece of cardboard in the door to leave a small opening promoting enough air exchange with the outside conditions (managed by the dehumidifier and the fan), which must be kept as cold as allowed by the AC unit. This approach, however, must be monitored closely as it may promote unstable or undesirable temperature conditions inside the units.

-Re-establishing Activity:

Towards the end of the desired brumation period, the reverse protocol was employed to "wake up" the animals. Temperature was gradually raised from 5ºC to 15ºC inside the chambers. Additionally, while animals remained under natural photoperiod, the amount of time that the basking lights were on was gradually increased. After 2-4 weeks of this gradual increase, the animals were returned to individual (males) or communal (females) terraria with access to bricks for refugia, water dish, 150W IR basking and UVB lights (Fig. 5.6). Again, the basking period was also gradually returned to the 8-10hour period supplied before brumation, as was the food supply (gradually increased from once a week to once every other day). The latter was done based on the animals' response when feeding. If the animals were seen to be active and readily taking food, then the regularity of feeding was increased. Animals were fed with multi-vitamin dusted crickets (*Acheta domesticus,* preferred option) or mealworms (*Tenebrio molitor*). Standing water was provided ad libitum by a water dish in each terrarium, and the terraria misted daily/ every other day.

Fig. 5.6: Set-up of the individual and communal terraria used to house the animals before and after brumation. Array of infrared heat/ basking lamps also visible.

5.8. After Brumation:

Animals were kept in the incubation chambers from the beginning of December 2020 to the end of March 2021. This is very likely a shorter period than that what they would usually brumate in the wild. This was due to the fact that these animals were still undergoing some tests at the time they would typically initiate brumation and then they still had to be slowly acclimated into brumation conditions. Furthermore, the decision was taken to "wake them up" from brumation earlier than what would naturally occur for several reasons namely the fact that this was the first time these conditions were devised, and it was thus far unknown if the conditions were right and if the animals were in fact losing condition or not, beyond their [encouraging] physical appearance. Additionally, it was also the intention that, as soon as conditions in the wild were favourable, the animals could immediately be returned to their site of capture in the best body condition possible. Hence a few weeks were taken to ensure the animals were well fed, properly active and in good condition to be released.

-Post-Brumation Body Condition and Mortality:

All animals survived the brumation period and emerged in good body condition with no noticeable change in the body condition before vs after brumation (Fig. 5.7). However, a few (3-5) weeks after the end of brumation some unexpected mortality was observed. 1 male *Iberolacerta monticola* died of unknown causes as well as some (6 from both species in total) of the females died from apparently a multitude of causes: some appeared to have developed fungal-like growths, while others were simply found dead, buried in the substrate of the communal terraria or under the tiles but with no signs of being physically trapped by the terrarium's hardscape.

Given that it was not possible to perform any necropsy or pathological analyses on the dead animals, precise cause of death was impossible to precisely pinpoint for these animals. The conditions (housing, food, environmental) in which the animals were housed post-brumation matched exactly those in which they had lived for circa 2 months prior to brumating, with no registered incidents of disease, death or even loss in condition.

Furthermore, the animals appeared in good condition (visual inspection for females, visual inspection and tracking of the weight of the males) and were checked at least once a week. On all such cases, death was quite sudden and unexpected. Nonetheless, a study by Alves de Matos et al., (2013) has shown (in these and other Iberian lacertid species) and increased incidence of iridovirus-like viruses in post-brumating lizards,

affecting their survivability. Although this was not monitored during the development of this protocol, it could explain the sudden susceptibility (and death) of the animals post-brumation and further reiterates de importance of good biosecurity and hygiene protocols.

Hence, for future applications of this protocol, it can be advisable to keep all the animals in individualised and simple (i.e. just water dish, refugium and basking platform, no soil) terraria post-brumation. This would make it easier to maintain a more sterile environment as well as to track each individual's feeding drive and health progress over the course of a few weeks (before contemplating further experiments or release), thus enabling easier containment of potential diseases that could spread across the captive population.

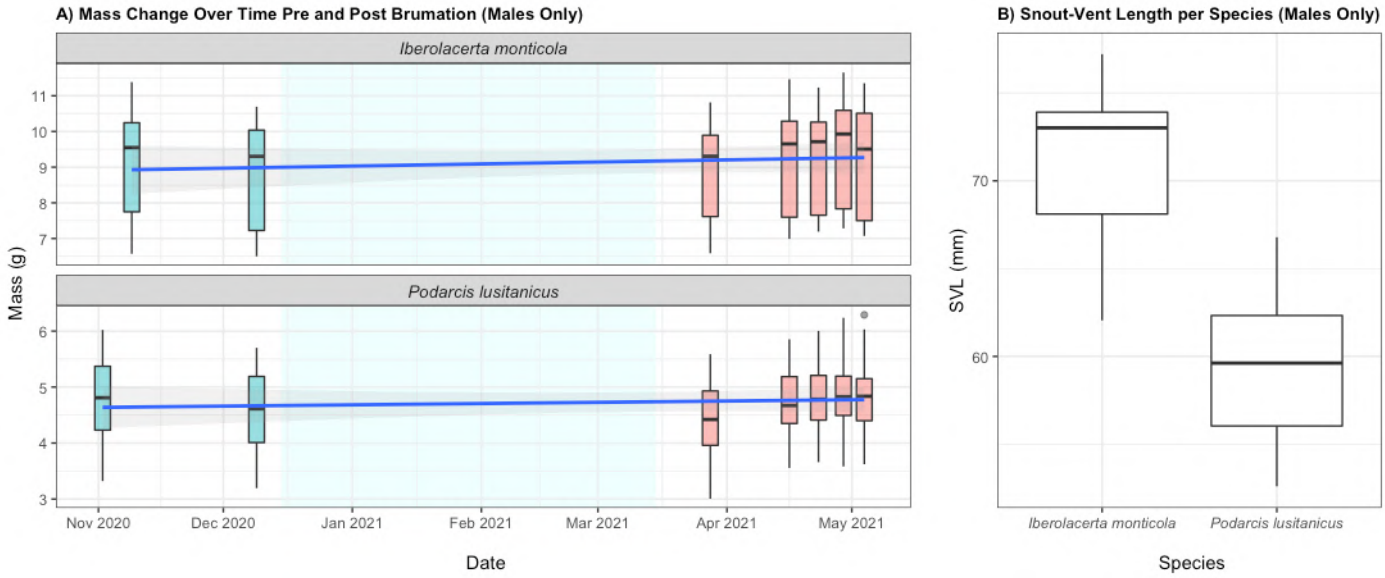

Fig. 5.7: A) Mass change over time for males of both species showing no difference between pre (blue) and post (red) brumation mass. Light blue shaded area corresponds to the brumation period; **B)** Snout-to-Vent lengths of males of both species showing the difference in size between the species.

5.9. Bioactive Substrate

The composition of the bioactive substrate described here is mostly based on personal experience and inspiration from the terrarium/ reptile husbandry community. This substrate system has proven functional on many occasions and for a range of applications namely as substrate for terraria as well as substrate for lizard nest boxes (Boskovits & Barroso et al., *in preparation*).

-Composition:

The (approximate) composition of the substrate is as follows (all percentages relate to volumes):

- 30% [hydrated] coconut husk (terrarium grade, usually comes dehydrated so water must be added prior to use)
- 30% organic potting soil (here it is important to make sure that there are no additives - pesticides, herbicides or chemical fertilisers - on the chosen soil)
- 20% sphagnum moss* mulch (often found in garden centres, for orchids and other epiphytes. Again, must ensure there are no additives and may need to be rehydrated before use)
- 10% fine silica-based sand (not sea/beach sand as this would have salt, and not calcareous sands – although this depends on the species being kept. The ideal is the “washed river sand” commercially available for mixing with cement, or the sand used for swimming pool filters – these are clean, inert and more economical than terrarium-grade alternatives)
- 10% crushed leaf litter/organic matter (could be collected from a garden as long as there are no pollutants or chemicals being used, must then be washed - ideally boiled for 15 minutes - and coarsely crushed/shredded. Leaves from toxic plants must also be avoided)

To create the substrate system, the components are thoroughly mixed and water is added until the soil has a moist/fresh consistency (without being wet or soggy; Fig. 5.8). After mixing all the components, the substrate was placed in an oven at 80ºC, distributed in flat trays, for a 1-3 hours (until fully dried) in order to ensure that any living organism (potential parasites, invertebrates, seeds/fungi that could germinate/grow in the soil) was killed. The procedure was carefully monitored to ensure nothing burned/combusted. Naturally, this also dehydrated the substrate, hence, after cooling, the soil must be rehydrated once again (ideally with dechlorinated water) into the above described condition.

***Note on ethical considerations**: given the ecological importance of sphagnum moss, and the general unsustainability of its over-exploitation, it is important to consider the conscientious use of this resource – especially among the terrarium/ reptile husbandry community. There is an increasing awareness of this impact with an equally rise in alternative, more sustainable materials. Hence, please consider trying a more sustainable alternative material to retain soil humidity and adjust the composition as needed.*

-The "Clean-Up Crew":

It is essential to colonise the soil with a "clean-up crew" to maintain soil health, degrade organic matter and prevent fungal growth. Isopods and collembola are the staple of such crew but other invertebrates such as earthworms may be considered depending on the desired outcome, conditions of the set-up, and species being housed. Nevertheless, for the collembola and isopoda used in the deployments of this protocol, so far, two acquisition options were considered:

1) Purchase from terrarium shops/suppliers - both collembola and isopods are widely available as "clean-up crew" for terraria in the pet trade. Issues may arise from the fact that these tend to be tropical species and hence may require higher temperatures and relative humidity in order to prosper. Furthermore, if these are to be deployed on terraria keeping animals that will later be released in the wild, there is an inherent risk of inadvertently introducing these non-native species into the wild (Jaskuła et al., 2019);
2) Collect them from the wild (ideally from the lizards' native range - Note: always consider potential permit requirements) - this would be the preferred method mostly due to the fact that the lizards kept in the lab are wild animals which will at some point be returned to the site of capture. Hence, by using a native clean-up crew one reduces the risk of potentially introducing non-native species to the wild. Additionally, using local species would mean that there is a higher chance that they would already be adapted to the thermal conditions that they would be exposed to, and hence increase potential survivability as well as maximal rate of "cleaning services".

   To collect isopods from the wild, simply search under decomposing wood, in a damp, shaded area, where they should be easy to find.

To collect collembola, several methods are described by terrarium keepers, most of which did not prove very efficient in my trials (pers. obs.). In the end, my own and preferred method relies on getting some large pieces of porous ["activated"] carbon (as used in the aquarium hobby) or lumps of coal (for barbecues), washing it thoroughly and soaking it in dechlorinated/drinking water for 10 minutes. These were then placed inside a perforated plastic box – the "collembola trap" - with many small (~2mm) holes (but not on the lid, to protect it from flooding with rain). The trap must then be deployed on the ground/in the leaf litter, in a shaded and humid natural(-ized) (e.g. garden, forest, etc.) area (or ideally, next to rotting wood or around compost heap), with no/low incidence of chemical pollutants (away from roads, pesticides, etc.), where it must be left for a couple of weeks. Collembola (and other small, beneficial, detritivore invertebrates) will naturally colonise the lumps of coal, these can then be mixed in with the substrate mix, effectively making it bioactive.

-General Maintenance:

The soil should be kept in a container with a perforated lid that allows breathability but also maintains some humidity. It is then very important that the soil is maintained well aerated (by frequently turning it around) and at an optimum hydration level (moist, not wet/soggy nor dry). The latter should be achieved by regular spraying ideally with dechlorinated water (or tap water left to stand in the air for 24-48hr). This is important for the good health of the clean-up crew (collembola and isopoda). If kept bio-active in the long-term, some calcium should be made available for the isopods to be able to thrive. This can be achieved by periodically adding a washed and shredded cuttlebone or reptile calcium supplement (as available in pet shops). The soil may need regular top-ups of clean-up crew, especially if left unattended for extended periods of time (e.g. if it dries out).

After use, the soil can be recycled indefinitely (somewhat – may need occasional top-ups of [sterilized] organic matter/ leaf litter). However, between uses, it should always be sterilised (by baking it at 80ºC) before being re-mixed in with the clean batch (where it will be recolonised by the clean-up crew). Whenever possible, the used soil should also be cleaned of as many "foreign bodies" as possible, such as visible faeces, shed skins, etc. This is mostly to avoid any scents or chemical cues potentially affecting the future users of the substrate.

Note: In case of mite (or any parasite/fungal) outbreaks, remove and discard all the substrate from all terraria and from the stored batch. Ideally, clean and disinfect everything (including the container as well as any utensil – e.g. shovel) very thoroughly and then re-start the bioactive substrate and colony from fresh components only once the outbreak in the collection has been fully eradicated.

Fig. 5.8: Photo of the bioactive substrate in its ideal hydration level.

5.10. Acknowledgements

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6. Chapter 6 – General Discussion and Conclusion

6.1. Discussion

Body temperature has long been a central variable in the study of ectotherm ecology, physiology and conservation biology, underpinning research on thermoregulation, physiology, performance, habitat quality, responses to environmental change, etc (e.g. Bennet et al., 1986; and reviewed extensively, for instance, in Angilletta, 2009; and in Taylor et al., 2020). Classical frameworks in thermal biology have emphasised the importance of linking body temperature to ecological and fitness-relevant processes, often through the use of standardised metrics such as preferred temperature, operative temperatures, thermal performance curves, etc. (e.g. Angilletta, 2009; Bakken, 1976; Huey & Slatkin, 1976; Huey & Stevenson, 1979), as discussed in this thesis' General Introduction. Each of these provide different yet important contributions to the field of ectotherm thermal ecology, still to this day.

However, as highlighted further in the General Introduction, most of this work has inevitably been constrained by the tools and methods available to measure temperature (be it of the animal's body or of its environment) at the time when said metrics were devised. In an important effort to ensure cross-comparability and standardization across the field [of reptile thermal ecology] many of such tools (e.g. thermocouple probes) and methods (e.g. cloacal temperature measures) have been, and still are, widely and ubiquitously used, only to become standards to which many recent studies still abide (reviewed in Camacho & Rusch, 2017; and Taylor et at., 2021). Yet, although many such methods, tools and metrics continue to hold a very valid place in this field of studies, under the bright light of the much-expanded current technological repertoire, recognising the possibility/ability to investigate (even address) some of the limitations or assumptions inherent to some of the standard/traditional measures and tools is highly prioritary (e.g. Barroso et al., 2016; Tattersall & Cadena, 2010). For instance, many standard metrics (e.g. T_{pref}) necessarily relied on single measures of temperature (e.g. the "grab and jab" approach - Avery, 1982 - and cloacal temperature as "quasi-dogmatic" proxy of general internal thermal state) and simplified metrics (e.g. T_{pref}) whose validity and interpretation

relies on assumptions – frequently untested (e.g. Hertz et al., 1993; vs Seebacher & Shine, 2004) – about heat exchange dynamics, organismal traits (e.g. surface-core gradients and regional heterothermy, or assumed lack thereof), as well as on the behavioural, social, physiological and environmental contexts and trade-offs (or, again, assumed lack thereof).

Thus, the works presented in this thesis revolved around the non-trivial question of "what is THE body temperature of an animal?", addressing some of its methodological underpinnings and the conceptual implications of assuming (instead of testing) its answer. The thesis built directly on the updated contemporary technological repertoire, and on recent developments in the field of ectotherm thermal ecology, examining how novel tools and approaches used to measure temperature will perform and compare across (and within) taxa, body sizes, environments (field vs laboratory, terrestrial vs amphibious, etc) and experimental contexts. Additionally, this thesis aimed to highlight the expanded toolkit now available to the thermal ecologist, in the hopes of teasing an interest in further investigating previously challenging assumptions as well as in promoting a seamless integration of the updated toolkit in an otherwise well-established (and well-standardized) field. Hence, the aim was not to cast a shadow over traditional tools and metrics, but to promote a deeper understanding of them, thus allowing for a more informed interpretation over said metrics and thermal ecology data in general. In other words, rather than rejecting the "old ways", this thesis calls for a context-aware perspective, treating proxies, standard/traditional metrics, tools and measurement techniques as conditionally valid representations of thermal state, while always acknowledging the value of cross- and retro-comparability of data in contributing towards a unified research field.

Across the chapters, surface, subcutaneous, and internal temperature measurements were evaluated as distinct but related measures, shaped by body size, thermal inertia, anatomy and physiology, behaviour, and environmental exposure. Furthermore, throughout this thesis, the contemporary toolkit is widely explored, testing the contexts under which such novel tools and methods may be additive to the field. This approach aligns with recent demands for greater methodological transparency and mechanistic grounding in studies of thermal ecology and ecophysiology, while inevitably circling the fundamental questions of "what is THE body temperature (can there even be a "chosen one"?) of an animal" and what is the best way (tool and/or method) to measure it.

By combining calibration experiments, high-resolution thermal imaging approaches, contact thermometry and internal temperature logging, this thesis demonstrates how

careful matching of tools to questions can improve both the accuracy and interpretability of thermal data. As shown in Chapters 2 and 3, in small squamates, non-invasive methods, such as thermal imaging, can yield reliable estimates of [internal] body temperature and patterns of regional heterothermy, when their limitations are explicitly addressed and due calibrations are considered (Playà-Montmany & Tattersall, 2021). In larger-bodied and more thermally buffered systems, such as those of large crocodilians explored in Chapter 4, internal measurements provide critical insight into complex thermal dynamics, while surface-based tools retain value when interpreted within an appropriate mechanistic framework. Hence, former chapters support the use of radiometric tools to infer the thermal state of small, terrestrial lizards, aligning with traditional assumptions (that internal body temperatures should not differ extensively from surface temperatures in small ectotherms - Bell, 1980) and with other thermographic studies in similarly sized reptiles (Barroso et al, 2016; Jones & Avery, 1989; Luna et al., 2013; Tosini & Avery, 1993). Contrastingly, the latter chapter does not find the same usefulness of this tool for inferring internal body temperature of the amphibious and large-bodied *Crocodylus moreletii*. This difference serves as a cautionary tale reiterating the importance of matching tool choice to the specific question and system under study. Collectively, these results reinforce the view that no single metric or method captures thermal biology in all contexts (Camacho & Rusch, 2017), but that robust inference emerges from understanding how different tools and measurements relate to one another (McMaster & Downs, 2013; Tattersall, 2016; Taylor et al., 2020).

Nevertheless, it is important to consider why cloacal temperature has historically become a *de facto* standard. This can be mostly attributed to its practical and relatively non-invasive (comparatively to intraperitoneal or hypothalamic measures, used in some studies - e.g. Bassetti et al., 2014; Hammel, Caldwell & Abrams, 1967) nature (Gans & Pough, 1982). This ease of measurement facilitated cross-study standardisation. However, its suitability as a general proxy of “internal” body temperature depends on physical and biological factors that vary widely among reptiles and contexts. From a heat-transfer perspective, the cloaca is not intrinsically closer to “core” temperature than other internal sites (Cremer et al., 2019; McMaster & Downs, 2013) or the neurological centres of thermoregulation (i.e. hypothalamus - Bartholomew, 1982; Crawford, Palomeque & Barber, 1977; Firth & Turner, 1982; Sannolo et al., 2014; Webb, Johnson & Firth, 1972); it is simply one easily accessible internal compartment. Nevertheless, its relationship to

deeper tissues inherently depends on surface-core distance (and hence respective thermal gradients), time constants, and perfusion (Clarac & Quilhac, 2018).

Body size is therefore critical: as mass and thermal inertia increase, spatial gradients - namely surface-to-core gradients and patterns of regional heterothermy - can become more pronounced and equilibration slower (e.g. Bell, 1980; McMaster & Downs, 2013; James & Mrosovsky, 2004), such that a temperature measured in the posterior body cavity may lag the thermal state of more centrally located tissues or important thermoregulatory (or thermosensitive) organs (e.g. brain) (Clarac & Quilhac, 2018; Bakken, 1976), as shown in Chapter 4 of this thesis. Nevertheless, it is important to note that small ectotherms, even when placed in constant/ homogeneous thermal environments (e.g. climactic chamber), can still exhibit patterns of regional heterothermy as well as different heat exchange dynamics according to size, shape and blood flow (Cox et al., 2023; Le Galliard et al., 2021a; Sannolo et al., 2014).

Geometry adds a further layer. In elongate body forms such as snakes, the cloaca may be far removed from functionally important tissues or thermosensory integration centres. Hence, longitudinal temperature gradients can be maintained over ecologically/physiologically relevant timescales, particularly during transitions between heat sources or microhabitats (Cox et al., 2023; Cox et al., 2024; Dzialowski & O'Connor, 1999; Dzialowski & O'Connor, 2004). Even among squamates, variation in body plan (stocky vs elongate) and posture (e.g. coiled vs stretched), insulation, and perfusion can alter the relationship between cloacal temperature and that of other internal compartments (Angilletta, 2009; Ayers & Shine, 1997; Stanton-Jones, Parusnath & Alexander, 2018), especially during rapid heating or cooling. These issues do not imply that cloacal temperature is "wrong", but rather that it is conditionally informative.

Ecology and thermoregulatory strategy can further decouple cloacal temperature from other internal or functionally relevant temperatures (Cremer et al., 2019; McMaster & Downs, 2013). As also demonstrated in Chapter 3 of this thesis, the relative importance of radiative versus conductive heat gain (e.g. heliothermy vs thigmothermy) influences where gradients develop – e.g. surface-to-core under strong radiation, or ventral-to-dorsal and/or along-body gradients under strong substrate coupling - thereby shaping what any single internal site represents (Bakken, 1976; Angilletta, 2009). Mode of life similarly matters: terrestrial, aquatic, and amphibious systems differ in boundary-layer properties, convective regimes, and the potential for rapid environmental heat exchange (Bell, 1980), altering the degree and persistence of internal–external decoupling (Bakken, 1976). In larger-bodied and semi-aquatic reptiles, for example, internal

temperatures may reflect strong buffering and time integration (Clarac & Quilhac, 2019), while peripheral or posterior compartments respond differently depending on circulation and exposure, as highlighted in Chapter 4. Viewed through this lens, the key methodological implication - reinforced by the work presented in this thesis - is not that cloacal temperature should be abandoned or replaced by a new "universal standard" (as one is unlikely to exist, anyway), but that it should be treated as one [albeit important] of several internal signals of thermal state, whose interpretability depends on organismal size, geometry, ecology, anatomy and physiology (e.g. McMaster & Downs, 2013), as well as the specific thermal process or state one aims to quantify. This also explains why there is unlikely to be a universal "chosen one" and emphasizes the importance of explicit calibration in order to attain robust inference and cross-comparability, careful matching of measurement body site to question, and transparent acknowledgement of what the chosen proxy can and cannot represent. Thus, it is precisely this "explicit calibration" that is the main (albeit not sole) take-home-message of this thesis.

Within the conceptual framework outlined above, the chapters of this thesis should be understood less as independent empirical studies and more as complementary methodological interventions addressing different facets of the same problem: how body temperature is defined and measured, and what can it tell us about an ectotherm's [thermal] ecology (i.e. how we interpret it), especially in the face of a much-expanded toolkit formerly unavailable to early seminal studies on this field. Each chapter targets a set of tools, deployed on different organisms and/or experimental contexts to evaluate how temperature measurements relate to organismal thermal state. Together, they contribute to the broader objective of developing, testing, and deploying methods that improve the interpretability and comparability of thermal data.

Despite widespread use of IRT in some systems (e.g. Tattersall & Cadena, 2010; Vollmer & Möllmann, 2018; Williams, 2024), a recurring critique has been the lack of robust, anatomically grounded calibration against more ubiquitous internal measures (e.g. cloacal temperature), limiting cross and retro-comparability across studies (Tattersall, 2016). Chapter 2 builds on previous studies (Barroso et al., 2016) providing further evidence that, in small squamates, surface temperatures derived from specific anatomical regions can track cloacal temperature closely across a range of thermal conditions. Specifically, it found that for *T. mauritanica*, unlike for lacertid lizards (Barroso et al., 2016), most of the tested surface regions correlated strongly ($R^2 > 0.87$; Spearman > 0.95) with cloacal temperature, indicating present but less pronounced patterns of regional heterothermy and smaller spatial heterogeneity in surface–internal

relationships. Nevertheless, the eye consistently provided the closest-to-isometric relationship (i.e., best slope/intercept combination), reinforcing it as a practical 'thermal window' (Barroso et al., 2016) when the goal is internal inference rather than surface mapping.

Thus, Chapter 2 emphasizes when and how infrared thermography may be an adequate, less invasive alternative to contact thermometry, and what pitfalls to avoid in order to ensure correct inference and cross-comparability of data. It shows how thermography can be an important tool that, given appropriate calibrations, approximates internal temperature. It also highlights the importance of considering patterns of regional heterothermy, which traditional single-point measurements, often overlooked in smaller animals (Barroso et al., 2016; Carretero, 2012), when considering body temperature estimates. Furthermore, in conjunction with Chapter 3 and in line with similar warnings from other "pro-IRT" thermal ecologists, it reiterates the importance of considering and correcting for additional parameters (e.g. emissivity, distance, etc.) for correct inference of IRT-measured temperatures (Faye, Dangles & Pincebourde, 2016; Playà-Montmany & Tattersall, 2021; Tattersall, 2016; Taylor et al., 2020).

Methodologically, this work also contributes with an updated and flexible calibration framework that can be adapted to different species and experimental settings to assure that all-important cross- and retro-comparability of body temperature measures. At the same time, while it makes the case that IRT-measured eye temperature may be a widely ubiquitous adequate proxy of internal thermal state - particularly in small reptiles - it reiterates the important fact that calibration relationships cannot be taken as transferable by default. Species-specific anatomy, posture, behaviour, and environmental conditions constrain generality (Playà-Montmany & Tattersall, 2021), leaving an open question of how broadly such frameworks can be extended across species, morphologies and ecological strategies as well as whether the "eye-as-window" claim (in small lizards) still holds true under conditions that deliberately stress emissivity/geometry assumptions (e.g. different integument types, distances/angles, and microclimate reflectance).

Although many thermal studies still rely on static correspondence between measurements, biophysical theory predicts that heating/cooling rates and mode of thermal exchange shape what any temperature signal represents (Bakken & Gates, 1975; Seebacher & Franklin, 2005). Chapter 3 builds on the foundations set by its predecessor but shifts the attention from static correspondence between measurements toward the processes that generate thermal signals. By providing a replicable set-up to explicitly quantify rates of heat gain and loss under different modes of heat exchange,

this chapter provides a stepping-stone towards a mechanistic bridge between IRT-measured surface temperature patterns and biophysical theory. More specifically, using radiometric video and a standardised workflow, this chapter showed that the same absolute surface temperature could arise from contrasting exchange histories (radiative vs conductive gain; heating vs cooling), producing body-region-specific rates that diverged most strongly early in trials - exactly when thermal gradients and exchange fluxes are expected to peak.

Furthermore, the reliance of the proposed set-up on radiometric video exposed yet another methodological necessity. While there are a number of tools, open source (e.g. “Thermimage” R package - Tattersall, 2021) or otherwise (e.g. “FLIR Tools” or “FLIR Thermal Studio Suite”, Teledyne FLIR LLC, Wilsonville, Oregon, USA), that enable easy and fast (even batch-) processing of radiometric photographs, these tools seldom apply to radiometric video processing. Furthermore, there is a general lack of protocols and best practices to guide a standardized processing of the radiometric video files, such as those resulting from the set-up devised in Chapter 3. Hence, as a by-product of this work, and of further deployments of the set-up devised here (Barroso et al, in preparation), an illustrated, stepwise protocol for manually processing radiometric videos using FLIR TOOLS+ (Teledyne FLIR LLC, Wilsonville, Oregon, USA) was also published in an open source repository (Barroso, 2024). Nevertheless, this manual processing remains both extremely laborious and subject to human bias, stressing the need for an automated, openly available solution. The problem was three-fold, as the tool in question would need to (i) be able to track specific, user-defined body locations (e.g. the eye); (ii) do so in a radiometric video (in itself a very complex issue – especially given that when a location, or the entire animal, is isothermal with its surroundings it becomes effectively indistinguishable from these); and (iii) extract, from the radiometric video, the associated temperature value of the tracked location(s).

Yet, although there are plentiful solutions, commercial (e.g. Ethovision XT - Noldus Information Technology, Wageningen, Netherlands) or open source (e.g. ReptiLearn - Eisenberg & Shein-Idelson, 2024), that allow tracking of animals in video recordings, equivalent tools for radiometric tracking were scarce or inadequate. The ones available were often designed with endotherms in mind and hence relied on thermal thresholds to define the tracking area (Gonzalez et al., 2016). For instance, the eye or the snout/nostrils of mammals and birds is often at a predictably different temperature than that of its surrounding tissues, or the whole animal itself is warmer than its surrounding environment. Hence a threshold of temperature can be defined for the software to identify

the body part or the animal it must track (Demartsev, Manser, & Tattersall, 2022; Jerem et al., 2015; Tabh et al., 2021). Such constant and predictable thermal differentials do not apply to ectotherms, as demonstrated in Chapter 3.

Hence, through the collaboration with the Mechanical Engineering Department from the University of Minho (Portugal), the LIACC (University of Maia, Portugal) and Centro ALGORITMI (Guimarães, Portugal), an ad-hoc software tool was developed to address this major hurdle. It relies on a stereo-video system that simultaneously records both radiometric and RGB videos, mapping one onto the other (pers. obs.; Afonso, Lopes & Ribeiro, 2024). The software then tracks the animal's body part in the RGB video and extracts the corresponding location's thermal value from the mapped radiometric counterpart. It relies on artificial intelligence, through the YOLOv5 machine learning algorithm, to automatically identify and track the measuring location (Afonso, Lopes & Ribeiro, 2024). The tracking algorithm has been trained with a range of lizard species, representing different sizes and geometries (pers. obs.), yet the large diversity of shapes and sizes of Squamata leaves much space to further train and improve in this tool, still in its infancy. Nevertheless, this tool – to be openly shared - represents another important methodological by-product of this thesis, addressing an emergent gap in the current thermal ecologist's toolkit and illustrating how new thermal tools often require parallel advances in data handling and automation before they can be adopted widely and reproducibly.

Additionally, a key conceptual advance that distils from this work is not the identification or [further] validation of a new proxy, but the demonstration that understanding *how* temperatures change (across time and across the animal's body) is often as important as knowing *what* temperature is measured. This process-based perspective clarifies why identical surface temperatures may reflect different internal states depending on heating regime, posture, substrate coupling, etc. Nevertheless, a limitation inherent to this approach is its reliance on simplified experimental conditions which, although flexible, modular and easy/cheap to replicate, may not be scalable towards much larger animals or those with habits beyond purely terrestrial.

Thus, scaling assumptions are particularly fragile in large-bodied ectotherms, where thermal inertia and spatial gradients can decouple internal compartments from external signals over biologically meaningful timescales (Bell, 1980; Clarac & Quilhac, 2019; McMaster & Downs, 2013). The central contribution of Chapter 4 is to demonstrate how body size, thermal inertia, anatomy and ecology may fundamentally alter the relationship between different thermal measurements. Additionally, it highlights how different tools

may be more or less adequate to accurately obtain said measurements, especially in a large and/or amphibious species. By integrating and contrasting thermographic with contact thermometry measures, and by comparing internal with surface and environmental temperature data in large-bodied reptiles, this chapter shows that calibration relationships observed in smaller ectotherms cannot be assumed to scale predictably. Specifically, this study found that IRT-measured eye temperatures in a crocodilian were a poor proxy of cloacal temperature, contrasting to the patterns observed in lacertid lizards (Barroso et al, 2016) and in the gecko tested in Chapter 2. However, it also found a relatively weak relationship between cloacal and gastric temperature, underscoring that "internal" sites can diverge meaningfully in large-bodied, amphibious reptiles. Empirically, this could hint that even two 'internal' measures can partition different components of thermal state in large crocodilians, reinforcing that anatomical placement must be justified, not assumed. Yet, comparing these two measures with another internal temperature measure – such as intraperitoneal temperature, often used in crocodilians (Bassetti et al, 2014; Campos & Magnusson, 2013) – would solidify such a claim or clarify whether indeed either of these internal measures may correlate more strongly with *de facto,* average internal state. Still, from this work emerge two candidate, easily accessible body locations (to attach or implant a data logger/transponder) that provide good proxies of internal temperature (given adequate calibrations): subcutaneous axillary temperature and external neck (post-occipital scute) temperature. These results may have important methodological repercussions, including for future tool design and placement, as widely discussed in the chapter.

Methodologically, this work brings to light the important role that increasingly diminutive data loggers (and, by association, transponders) can play in thermal ecology studies (see, for example: McMaster & Downs, 2013; Perry, Gangloff & Rutschmann, 2025) for further examples), while conceptually underscores the necessity of spatially and temporally explicit calibration especially in thermally buffered systems. It also warns for caution against extrapolating indiscriminately proxy and tool performance across orders of magnitude in body mass and across habitat preferences and lifestyles. At the same time, it still leaves unresolved questions regarding the extent to which these patterns generalise across other large reptiles or ectotherms with different ecologies (e.g. insects), highlighting a clear avenue for much-needed future comparative and/or methodological work.

Finally, reproducibility and welfare constraints remain under-discussed methodological drivers in ecophysiology, even though pre-experimental history and husbandry can propagate into physiological measurements and among-study variance (Denommé, Bakker, & Tattersall, 2025). Chapter 5 contributes at a different, but complementary, level by addressing this oversight in the field. By focusing on brumation under laboratory conditions, as an example, this chapter calls out a gap in husbandry guidelines, in itself a methodological constraint that can influence both animal welfare and downstream physiological measurements, yet rarely standardised or explicitly justified (Beaupre et al., 2004). The contribution here is not a new measurement technique, but a structured, adaptable framework for brumating two lacertid species in the lab. More generally, it's a call for transparency and ethical reflection in husbandry standards, raising important open questions regarding how [pre-]experimental history – namely captive animal husbandry, particularly during low-metabolic states – may shape subsequent thermal responses, and how such effects might contribute to unexplained variability among studies.

Across chapters, several thesis-level take-home messages emerge that speak directly to the overarching objective of the thesis. Firstly, this thesis highlights the fact that, although some body locations may be more ubiquitously good "thermal windows" providing potential proxies of internal state, one must remember to abide by the fact that there is likely no singular or universally privileged measure of "body temperature" and hence system-specific considerations and calibrations are essential. It demonstrates that instead, different measurements capture different, context-dependent aspects of thermal state. Secondly, proxies and classical metrics remain valid and valuable, but their assumptions and limitations must be explicitly acknowledged and, if possible, tested – i.e. a call to action. Thirdly, calibration - across measurement types, body regions, tools, etc - is essential for robust thermal inference. Finally, methodological choices, including ethical and procedural (i.e. husbandry) ones, may shape not only data quality but also the comparability and interpretability of thermal studies and hence, there is an urgent need to devise adequate and standardised/standardisable guidelines on these aspects too. Together, these insights emphasise that advances in thermal ecology are driven not solely by new tools, but by how those tools are integrated, calibrated, and deployed within an existing conceptual and methodological framework.

These chapter-level insights also have a broader methodological implication: they help position new and traditional temperature tools within a common interpretive framework, rather than always as competing alternatives. Seen from this perspective, the question

is no longer whether new tools should be used in thermal ecology, but how they can be integrated coherently into an already well-developed and standardised field. Over the last two decades, the range of available approaches to measure temperature and thermal environments has expanded considerably. This expansion has enabled unprecedented spatial and temporal resolution in thermal measurements (Blais et al., 2023; Taylor et at., 2021), while simultaneously challenging long-standing assumptions about what constitutes a meaningful estimate of body temperature and how different measurements should be interpreted (Barroso et al., 2016; McMaster & Downs, 2013).

In practice, the modern thermal ecologist toolkit spans (i) contact measurements (cloacal probes; subcutaneous/intracoelomic loggers) (e.g. Bassetti et al., 2014; McMaster & Downs, 2013; Reider, Zerger & Whiteman, 2022), (ii) contactless radiometric approaches (spot IR thermometers; infrared thermography; radiometric video) (e.g. Chukwuka, Virens, & Cree, 2019; Tattersall, 2016; Tattersall & Cadena. 2010), (iii) environmental characterisation tools (operative temperature models including 3D-printed physical models; microclimate sensor arrays; thermal mapping) (e.g. Alujević et al., 2023; Blais et al., 2023), and (iv) integrative platforms (multi-sensor biologgers combining temperature with acceleration/activity, depth, posture, location, etc.) (e.g. Brown, Kays, Wikelski, Wilson, & Klimley, 2013; Waller et al., 2023), (v) controlled experimental systems (e.g. mesocosms, climatic chambers) (Bodineau et al., 2024; Le Galliard et al., 2021b; Saçdanaku, Nunes & Carretero, manuscript submitted; Simbula et al., 2025). Each class solves a different bottleneck, while still having their limitations: contact sensors increase confidence about internal state but raise invasiveness and welfare constraints (Cremer et al., 2019; Langkilde & Shine, 2006); radiometric tools increase spatial resolution and reduce handling, but require emissivity/geometry-aware calibration (Playà-Montmany & Tattersall, 2021; Tattersall, 2016); operative models provide null expectations and allow explicit thermoregulatory inference, but depend on how faithfully models reproduce radiative and convective exchange (Alujević et al., 2023) as well as inertial effects (Lutterschmidt & Reinert, 2012); and multi-sensor tags connect temperature to behaviour and exposure, but are constrained by size, attachment/implantation logistics, battery life, and data recovery (Brien et al., 2010; Franklin et al., 2009; Reider, Zerger & Whiteman, 2022, Robert & Thompson, 2003).

Importantly, many of these tools do not generate fundamentally new thermal variables, but instead provide alternative windows into the same underlying processes. Infrared thermography, for instance, yields spatially explicit surface temperature data that reflect radiative and convective heat exchange rather than internal temperature per se

(Tattersall & Cadena, 2016). Yet, capitalising on species-specific anatomical knowledge of "thermal windows" allows said data to be calibrated against internal temperature measures, ultimately providing non-invasive proxies of internal thermal state. Data loggers and transponders, by contrast, capture temperature at a fixed anatomical location and integrate environmental exposure over time, often smoothing short-term variability - particularly in small animals - in favour of longer-term trends (Taylor et al., 2020). Although still effectively a contact measure, their ability to log and/or transmit data remotely inevitably reduces the rate of direct interaction with the subject, thus potentially providing large amounts of data with minimal constraint or stress to the animal. A level above are multi-sensor tags which, although still slow to percolate into the field of reptile thermal ecology, allow temperature to be recorded alongside activity, depth, acceleration, or posture, facilitating more mechanistic interpretations of thermal data in relation to behaviour and habitat use (Kearney et al., 2021; Reyes-Puig et al., 2025). Still their [minimum] size, albeit rapidly decreasing, constrains their application possibilities (but see Perry, Gangloff, & Rutschmann, 2025). Furthermore, tightly controlled systems which can simulate specific environmental conditions across a range of timescales, such as climatic chambers and mesocosms, offer the ability to test, isolate or manipulate specific drivers of thermal variation (Le Galliard et al., 2021a), while operative temperature models provide null expectations against which thermoregulatory behaviour can be assessed (Christian, Tracy & Tracy, 2006; Seebacher & Shine, 2004). Each of these approaches samples a different component of the thermal system, and none can be considered intrinsically superior outside the context of a specific question.

One of the key opportunities created by this expanded toolkit is the ability to revisit classical metrics with greater nuance. Measures such as preferred temperature, operative temperature, or thermal safety margins remain central to thermal ecology precisely because they provide a common currency for comparison across studies and taxa (Angilletta, 2009; Taylor et al., 2020). However, non/minimally-invasive, high-resolution surface and internal measurements now make it possible to examine how these metrics emerge from underlying thermal heterogeneity and dynamics all while potentially diminishing the experimenter impact (Langkilde & Shine, 2006). For example, thermal imaging can reveal whether a preferred temperature measured at the cloaca corresponds to uniform internal conditions or masks substantial regional heterothermy, while continuous internal logging can show whether apparent preferences reflect stable equilibria or transient states shaped by recent thermal history. In this sense, new tools

do not undermine established metrics, but rather allow their assumptions to be tested explicitly and their biological meaning to be refined.

Beyond improving interpretation of temperature itself, the contemporary toolkit also facilitates the integration of additional physiological and ecological variables that interact with thermal processes. From a measurement perspective, these factors matter not only biologically but methodologically - for instance: if hydration state/ evaporative water loss rates or reproductive condition alter perfusion, evaporative cooling, or posture, they can shift patterns of regional heterothermy and surface–internal gradients, therefore potentially affecting the reliability of otherwise well-calibrated proxies. Yet hydration is rarely quantified alongside body temperature, nor is it usually accounted for when estimating thermal metrics (e.g. T_{pref} – but see Le Galliard et al., 2021a; and Bodineau et al., 2024), despite growing recognition that hydroregulation can constrain thermal choices and performance (Fernández-Rodríguez, Barroso, & Carretero, 2021; Rozen-Rechels et al., 2019; Sannolo, Barroso & Carretero, 2017; Sannolo & Carretero, 2019). Methodologically, this omission matters because dehydration can shift behavioural thermoregulation and alter the apparent meaning of standard thermal metrics (e.g., a lower “preferred temperature” may reflect hydric constraint rather than altered thermal optima), making temperature-only datasets harder to interpret mechanistically (Alomar et al., 2024; Anderson & Chapple, 2025; Brusch et al., 2020; Gómez-Alés et al., 2026; Plasman et al., 2025; Sannolo & Carretero, 2019). Advances in gravimetric methods (e.g. Clark & Nicholson, 1994; Dawson, Shoemaker & Licht, 1966; Sannolo, Barroso & Carretero, 2017), evaporimetry (e.g. Weaver et al., 2022; Weaver et al., 2023) and humidity sensing (e.g. Dos Santos et al., 2016; Weaver et al., 2023) now make it increasingly feasible to examine water balance and temperature jointly, opening avenues to reassess thermal preferences, limits, and safety margins under realistic ecological constraints.

Similarly, sex-specific and reproductive effects remain underrepresented in thermal ecology, even though differences in body composition and shape, hormonal regulation, and reproductive state can influence thermal inertia, perfusion, physiological priorities and behaviour (Pottier et al., 2021; Sinclair et al., 2016). Much of the existing literature is biased towards males, often for logistical convenience, raising questions about how broadly reported thermal metrics apply across populations composed of males, gravid and non-gravid females, immatures and eggs. As a result, “species-typical” thermal metrics often confound sex- and state-specific physiology with broader ecological patterns, which becomes especially problematic when datasets are pooled for studies

on macroevolution and macroecology (e.g. Camacho et al., 2024). Ontogenetic stage (e.g. Carretero, Roig & Llorente, 2005), nutritional condition/ feeding status (e.g. Brown & Griffin, 2004; Perry et al., 2025), and health status (e.g. Paranjpe et al., 2014; Scholnick et al., 2010), all shown to have an effect of T_{pref}, for instance, may also further modulate patterns of regional heterothermy and/or surface-core gradients, and hence proxy relationships, particularly in systems where energy or water balance is tightly coupled to temperature. Addressing these sources of variation will require both broader sampling strategies, improved experimental methodologies and the careful deployment of multi-sensor approaches capable of capturing interacting physiological processes alongside temperature.

6.2. Conclusion

Ultimately this thesis set out to develop, test, and deploy methodological approaches for studying thermal biology in reptiles, with an applied focus on squamates and crocodilians. As outlined in the General Introduction, the central aim was to examine how different ways of measuring temperature relate to organismal thermal state, and how methodological choices influence ecophysiological inference. Rather than advancing a single ecological hypothesis, the work is unified by a methodological objective: to clarify what different temperature measurements represent, under which conditions they are informative, and how they can be integrated within a coherent analytical framework.

Across chapters, the thesis demonstrated that no single measurement technique captures thermal biology across all reptile taxa, body sizes, and ecological contexts. Surface, subcutaneous, and internal temperature measurements provide complementary information, whose relationships depend on anatomy, thermal inertia, ecology, and recent thermal history. Furthermore, the evidence presented here demonstrates that, similarly to measurements discussed prior, no one tool – neither thermal cameras, nor data loggers/transponders, nor contact probes – far outweighs all others, reiterating that tool choice will be species, context and question dependent. In small terrestrial squamates, for instance, the results show that non-invasive radiometric tools such as IR thermography and videography can, when appropriately calibrated, replace more invasive contact methods for specific questions, while also provide the added benefit of revealing spatial and temporal thermal structure that single-point measurements cannot resolve. In larger-bodied, amphibious crocodilians, however, data loggers proved more useful and reliable than thermographic measures of body

temperature. Together, these findings directly address the questions posed in the General Introduction regarding the definition and measurement of body temperature and the conditions under which different tools can be useful and meaningfully compared.

Thus, the central contribution of this thesis is therefore methodological. Contemporary tools such as infrared thermography, radiometric video, internal loggers (and, by extrapolation, emerging multi-sensor tags) are shown to be neither universally superior nor merely complementary by default. Their value lies in how they are calibrated, deployed, and matched to the biological process of interest. In some contexts, modern tools can replace traditional approaches; in others, they extend or contextualise them. Progress in thermal ecology thus depends less on the adoption of new technologies per se than on the development of transparent, reproducible workflows that explicitly link tools, measurement locations, and thermal processes.

The thesis also highlights that methodological considerations extend beyond sensors and instrumentation. Experimental design, data processing pipelines, and husbandry practices -including those associated with low-metabolic states such as brumation – have the potential to shape thermal measurements in ways that are rarely made explicit, yet directly affect data quality and comparability. Addressing these factors is therefore integral to improving ecophysiological inference, rather than supplementary to it.

In summary, this work strengthens the methodological foundations of reptile thermal ecology by demonstrating how contemporary tools can be critically evaluated, calibrated, and integrated within an established field. Beyond simply offering definitive answers to specific ecological questions, the thesis provides a critical framework that enables existing questions to be revisited with greater resolution, and new questions to be addressed with clearer understanding of what temperature measurements do - and do not - represent. Certainly, the next decade will see substantial steps forward in the development of thermal ecology studies. However, this will only be possible through the integration of novel technologies, with quantitative analyses and critical thinking, that is, by the implementation of the standards of the well-recognised Scientific Method.

6.3. References

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Appendix A – List of other publications during PhD:

Throughout the course of the PhD I have also been involved (some as lead/co-lead, others as a collaborator) in several other projects ultimately resulting in multiple other publications, which have either since been published or are currently in preparation. Several of these works have been complimentary, consequential, or further explorations/deployments of the tools and/or methods discussed in this thesis.

Hence, I hereby present a list of the additional published works for which I contributed during the course of my PhD years:

1. Simbula, G. & **Barroso, F. M**., Saçdanaku, E., Ene, G., Fănaru, G., Sreelatha, L. B., . . . Carretero, M. A. (2025). A cost-effective mesocosm framework for reptile research: Design, validation, and practical insights. *PLoS ONE*, 20(11), e0337616. https://doi.org/10.1371/journal.pone.0337616
2. Žagar, A., Dajčman, U., Megía-Palma, R., Simčič, T., **Barroso, F. M.**, Baškiera, S., & Carretero, M. A. (2024). Analysis of subcellular energy metabolism in five Lacertidae lizards across varied environmental conditions. *Comparative Biochemistry and Physiology Part a Molecular & Integrative Physiology*, 297, 111729. https://doi.org/10.1016/j.cbpa.2024.111729
3. **Barroso, F. M.** (2024). Illustrated Protocol for Processing Radiometric Videos Using the Software FLIR Tools : An Approach Applied to a Novel Methodology for Lizard Thermal Exchange Rates Studies v1. *Protocols.io*. https://doi.org/10.17504/protocols.io.n2bvj358xlk5/v1
4. Hastings, B. T., Melnyk, A., Ghyabi, M., White, E., **Barroso, F. M.**, Carretero, M. A., . . . Chiari, Y. (2023). Melanistic coloration does not influence thermoregulation in the crepuscular gecko *Eublepharis macularius*. *Biology Open*, 12(10). https://doi.org/10.1242/bio.060114
5. Fernández-Rodríguez, I., **Barroso, F. M**., & Carretero, M. A. (2021). An integrative analysis of the short-term effects of tail autotomy on thermoregulation and dehydration rates in wall lizards. *Journal of Thermal Biology*, *99*, 102976. https://doi.org/10.1016/j.jtherbio.2021.102976