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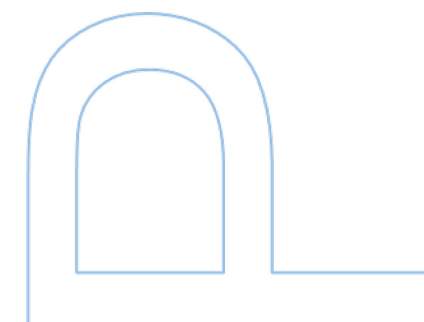
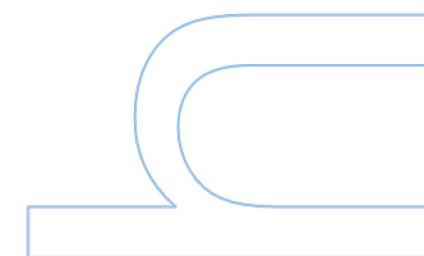
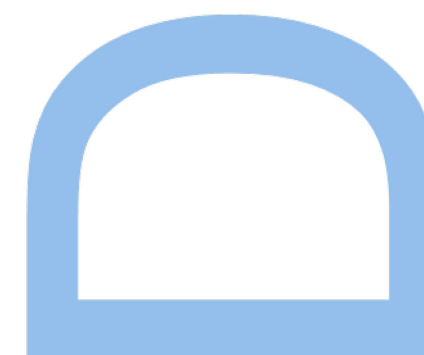
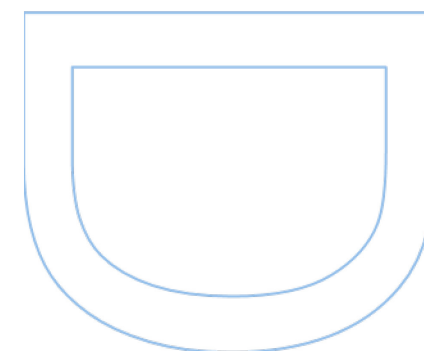
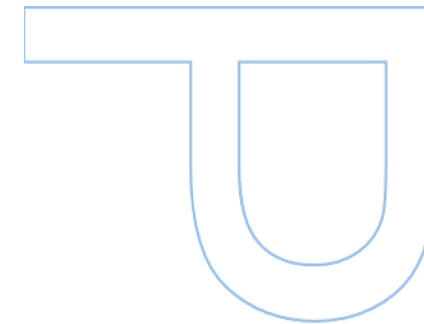
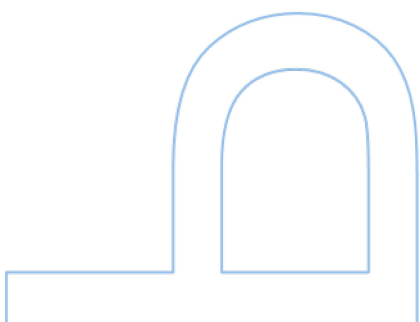
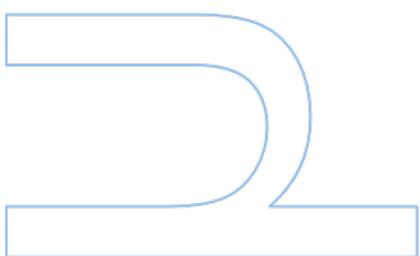
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evolution, gene dynamics and ecological drivers

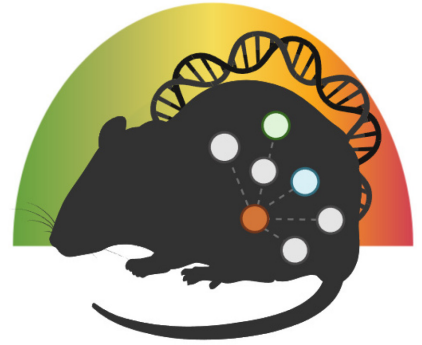
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Flexibility of thermoregulation in mammals: Molecular evolution, gene dynamics and ecological drivers

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Lisbon
2025





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

Faculty of Sciences of the University of Porto and Department of Biology of Freie Universität, Berlin

2025

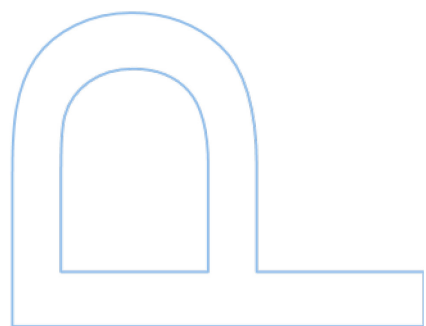
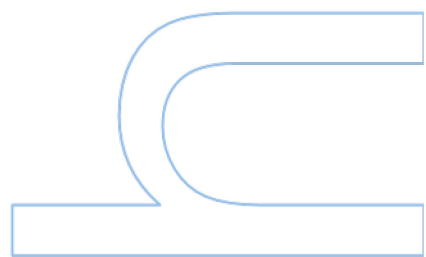
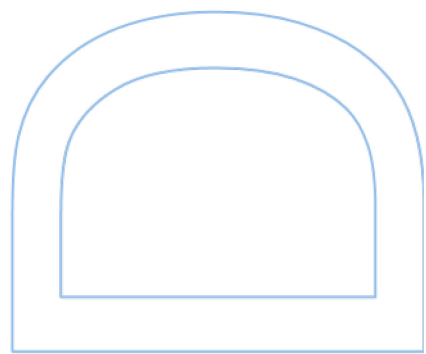
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Dedicated to all my loved ones

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"Sometimes science is more art than science"

Resumo

A endotermia, capacidade fisiológica de manter temperaturas corporais elevadas e estáveis, tem sido fundamental para o sucesso ecológico dos mamíferos, acarretando, no entanto, elevados custos energéticos. Em ambientes pobres em recursos ou com elevada variabilidade térmica, como desérticos ou árticos, estes custos podem tornar-se incompatíveis com a vida. A heterotermia, definida por reduções metabólicas e de temperatura corporal, oferece uma estratégia fisiológica para conservação de energia, para algumas espécies de mamíferos. No entanto, as suas condicionantes ecológicas, fundamentos moleculares e dinâmicas de desenvolvimento ainda se encontram pouco estudadas. Esta tese investiga a heterotermia como uma característica adaptativa multifacetada. Análises comparativas a 81 espécies de mamíferos revelam que a temperatura corporal mínima é fortemente determinada pela baixa produtividade do habitat e a elevada variabilidade térmica, particularmente em regiões húmidas. Contrariamente, espécies adaptadas a ambientes áridos apresentam temperaturas corporais mínimas diárias mais elevadas, indicativas de restrições ecológicas e fisiológicas. Análises genómicas a 21 espécies de roedores mostram que, embora os genes associados à termorregulação sejam amplamente conservados, espécies com maior flexibilidade térmica exibem uma seleção aminoacídica positiva maior, bem como uma integração em redes de interação proteína-proteína mais ampla. Isto sugere que a inovação evolutiva na termorregulação reside na complexidade da rede e não em alterações genéticas isoladas. A nível transcriptómico, as espécies com maiores amplitudes de flutuação de temperatura corporal apresentam redes de co-expressão mais interconectadas, especialmente em vias cognitivas e metabólicas. Esta reconfiguração reflete plasticidade adaptativa, revelando uma compensação entre a estabilidade fisiológica e a capacidade de resposta à variabilidade das condições ambientais. Finalmente, análises de desenvolvimento em ratinhos de laboratório demonstram que a endotermia surge progressivamente antes do desmame, ao dia 14 do desenvolvimento pós-natal, sendo que a poiquilotermia no início da vida representa uma fase de transição conservada. A temperatura materna modula a trajetória para a independência térmica, ao passo que a ausência de material de ninho ou o sexo não evidenciam efeitos significativos. Em conjunto, estas descobertas apoiam a heterotermia como um contínuo fisiológico, molecular e de desenvolvimento. Ao integrar dados ambientais, evolutivos, moleculares e fisiológicos, este trabalho fornece uma estrutura abrangente para a compreensão da adaptação térmica em mamíferos, realçando a relevância da flexibilidade na termorregulação como um fator chave para ultrapassar restrições energéticas e suportar a previsão da resiliência das espécies num clima em constante mudança.

Palavras-chave: termorregulação, heterotermia, endotermia, flexibilidade metabólica, redes genéticas, evolução de proteínas, temperatura corporal, fisiologia do desenvolvimento, adaptação climática, mamíferos, balanço energético, fisiologia ecológica

Abstract

Endothermy, the ability to physiologically maintain high and stable body temperatures, has been central to mammalian ecological success but comes at high energetic cost. In resource-poor or thermally variable environments, such as desertic or arctic, these costs can be unsustainable. Heterothermy, defined by reversible reductions in metabolic rate and body temperature, offers a physiological strategy to conserve energy, for some mammal species, yet its ecological drivers, molecular underpinnings, and developmental dynamics remain incompletely understood. This thesis investigates heterothermy as a multi-scale adaptive trait. Comparative analyses across 81 mammalian species reveal that minimum body temperature is strongly predicted by low habitat productivity and high thermal variability, particularly in humid regions. In contrast, arid-adapted species exhibit higher minimum daily temperatures, suggesting ecological and physiological constraints. Genomic analyses across 21 rodent species show that while thermoregulatory genes are broadly conserved, species with higher thermal flexibility exhibit stronger positive amino acid selection and greater integration into protein-protein interaction networks. This suggests that evolutionary innovation in thermoregulation lies in network complexity rather than isolated gene changes. At the transcriptomic level, species with greater body temperature fluctuations display more rewired co-expression networks, especially in cognitive and metabolic pathways. This rewiring reflects adaptive plasticity, revealing a trade-off between physiological stability and responsiveness to environmental variability. Finally, developmental analyses in laboratory mice demonstrate that endothermy emerges progressively before weaning, at postnatal day 14, with early life poikilothermy representing a conserved, transitional phase. Maternal temperature, rather than nest presence or sex, modulates the trajectory toward thermal independence. Together, these findings confirm heterothermy as a physiological, molecular, and developmental continuum. By integrating environmental, evolutionary, and molecular-physiological data, this work provides a comprehensive framework for understanding thermal adaptation in mammals. It highlights the importance of thermoregulatory flexibility in coping with energetic constraints and underscores its relevance in predicting species resilience in a changing climate.

Keywords: thermoregulation, heterothermy, endothermy, metabolic flexibility, gene networks, protein evolution, body temperature, developmental physiology, climate adaptation, mammals, energy balance, ecological physiology

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List of Abbreviations

AAAS	
American Association for the Advancement of Science	i
AI	
Aridity Index	18, 23, 96
AIC	
Akaike Information Criterion	15, 20, 21, 23, 24
Anc	
Ancestral	44, 46, 47, 48
ATP	
Adenosine triphosphate	33, 43, 44, 46, 47, 48, 49, 51
BM	
Body Mass	17, 93
CID	
Chemically Induced Dimerization	33
COST	
European Cooperation in Science and Technology	i
CPPS	
Conserved Positively Selected Amino Acid Sites	41
CSV	
Comma Separated Values	60
DGE	
Differential Gene Expression	58, 62
DNA	
Deoxyribonucleic Acid	58
FAG-EC	
Fast Agglomerate Algorithm	34
FASTA	
Fast-All	32
FCT	
Fundação para a Ciência e Tecnologia	i
FCUP	
Faculdade de Ciências da Universidade do Porto	i
FU	
Freie Universität Berlin	i
GFF	
General Feature Format	58
GO	
Gene Ontology	51, 59
HADDOCK	
High Ambiguity Driven protein-protein DOCKing	33
HET	
Heterotherm	39, 41, 44, 46, 48, 101, 102
HETATM	
Hetero Atom	33
HIB	

Hibernator	39, 41, 46, 48, 102
HO	
Homeotherm	39, 41, 44, 46, 48, 102, 103, 104
IL	
Interleukin.....	38
KEGG	
Kyoto Encyclopedia of Genes and Genomes	51, 59
LASA	
Laboratory Animal Science Association	i
LRT	
Likelihood ratio test.....	32
MAFFT	
Multiple Alignment using Fast Fourier Transform	32, 93
MSA	
Multiple Sequence Alignment.....	32, 33, 46
NA	
Not Applicable.....	20, 44, 46, 98, 99, 100
NAD	
Nicotinamide Adenine Dinucleotide.....	33, 46, 48, 49, 98
NCBI	
National Center for Biotechnology Information	38
NDVI	
Normalized Difference Vegetation Index.....	14, 15
NOD	
Nucleotide-binding and Oligomerization Domain-like	51
OLS	
Ordinary Least Squares.....	15, 18
PAML	
Phylogenetic Analysis by Maximum Likelihood.....	32, 97
PCA	
Principal Component Analysis.....	15, 23
PDB	
Protein Data Bank.....	32
PGLS	
Phylogenetic Generalized Least Squares	15, 18, 20, 23, 24
P _M	
Primary Productivity.....	15, 17
PPI	
Protein-Protein Interaction.....	33, 49, 50
PRODIGY-LIG	
PROtein binDIng enerGY prediction - LIGands	33, 97
P _{SD}	
Among-years variation in Productivity	15, 17
PSS	
Positively Selected amino acid Sites.....	32, 33, 34, 36, 39, 40, 41, 46, 48, 101, 107, 108, 109, 110, 111, 112
RC	
Redundant Component.....	20, 23
RMSE	
Root Mean Square Deviation of atomic positions	44, 46, 47, 48, 49

RNA	
Ribonucleic acid	51, 57, 60
SE	
Standard Error	21, 22, 23
SMBE	
Society for Molecular Biology and Evolution	i
SPCAL	
Sociedade Portuguesa de Ciências em Animais de Laboratório	i
TB	
Body Temperature	80
TS	
Superficial Temperature	14, 15, 17, 78, 79, 93
Tsc	
Subcutaneous body temperature	78, 80
Wt	
Wild type	44, 46, 47, 48
wTO	
Weighted Topological Overlap	59, 60, 64

Chapter 1. Main introduction

1.1. The problem: Energetic constraints under climate change

Endothermy is believed to be a key driver of the evolutionary success of mammals and birds, enabling their dispersion into diverse thermal environments (Lovegrove, 2003; Clarke & Pörtner, 2010). This physiology refers to the capacity to internally generate and conserve heat, maintaining a high and relatively stable core body temperature, despite varying ambient conditions (McNab 1978, Nespolo *et al.* 2011, Lovegrove 2017, Rezende *et al.* 2020). This thermal constancy improves and maintains optimal enzyme kinetics, complex neural processing, and sustained levels of activity and reproduction (Preußner *et al.* 2017). However, endothermic homeothermy physiology is energetically expensive, with endothermic mammals requiring five-ten times more energy than ectothermic reptiles of comparable size, solely to maintain basic life functions (Figure 1; Pough 1980; McNab 2009).

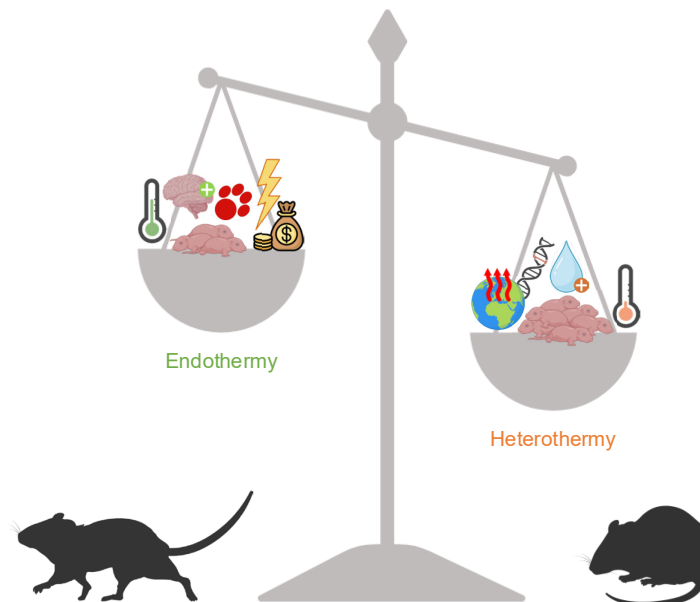


Figure 1 - Adaptive trade-off between endothermy and heterothermy in mammals. Physiological and ecological trade-offs between endothermy (left), marked by high cognitive capacity, thermal stability and costly metabolic performance and higher extinction risks, and heterothermy (right), which enables reversible metabolic downregulation, water conservation, and resilience to climatic variability and extinction events, higher reproduction success and fitness. The scale tilts towards heterothermy, reflecting its adaptive advantage under energetic constraints and environmental unpredictability.

These energetic demands become particularly limiting in environments with high thermal variability and limited food resources, such as deserts and semi-arid regions (Tieleman & Williams 1999; Vuarin & Henry 2014; Dammhahn *et al.* 2017). This is an important problem because in response to such constraints, heterothermy, which is a physiological strategy involving large, reversible reductions in metabolic rate, body temperature, and water loss, provides means for conserving energy (IUPS Thermal Commission 2003). Physiological flexibility, likely evolved as an adaptive response to environmental

unpredictability and resource limitation, enhances individual fitness (Geiser 2008; Geiser & Brigham 2012; Hetem *et al.* 2016; Lovegrove 2017). Heterothermy has been proposed as a trait that may enhance survival during historical extinction events and buffer populations against increasing climate variability (Lovegrove 2017; Geiser & Turbill 2009). Environmental variables, including ambient temperature and habitat productivity, have been empirically linked to the expression and extent of daily heterothermy across mammalian species (Bastos *et al.* 2021). Species with lower minimum body temperatures, often used as a proxy for heterothermic capacity, are typically associated with regions characterised by high interannual temperature variability and low resource availability (Bastos *et al.* 2021). These environmental contexts impose energetic constraints that render strict homeothermy metabolically costly and potentially unsustainable. Such physiological trade-offs are especially pertinent in climatically extreme and biodiversity-rich regions like the Sahara-Sahel, where ecological vulnerability is high and adaptive responses remain poorly understood (Brito *et al.* 2016). Despite growing recognition of heterothermy's adaptive value under thermal and energetic stress, further research is needed to understand how this strategy modulates energy and water balance across ecological gradients, and how it may enhance resilience to climate change.

1.2. The phenomenon: What is daily heterothermy?

Unlike strict homeothermy, which maintains relatively stable body temperatures, or hibernation which involves prolonged or seasonal torpor, daily heterothermy allows transient and reversible reductions in body temperature in response to environmental or energetic stressors, and which usually lasts less than 24h (Bligh & Johnson 1973, Boyles *et al.* 2011, Geiser & Brigham 2012, Vuarin & Henry 2014). Physiologically, daily heterothermy or torpor, which are short bouts characterized by significant reductions in metabolic rate and body temperature (Geiser 1988, Heldmaier *et al.* 2004). The importance of this phenomenon lies in the fact that it reduces both energy and water expenditure, providing adaptive advantages in environments marked by unpredictability and limited resources (Tieleman & Williams 1999, Vuarin & Henry 2014; Figure 2).

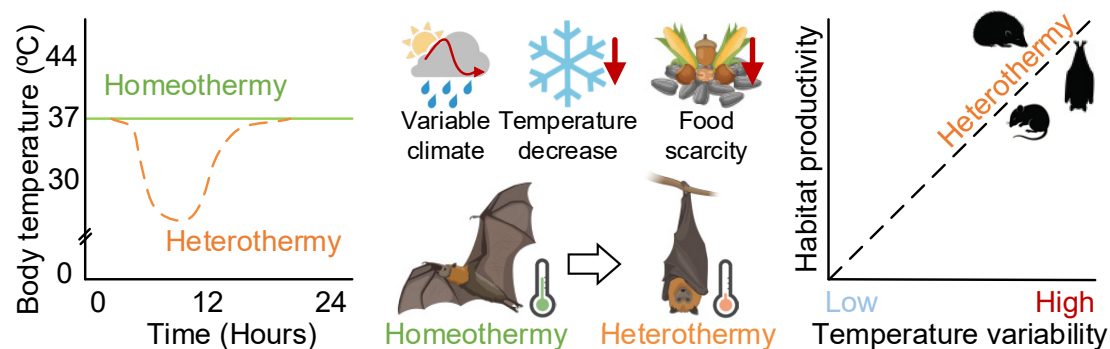


Figure 2 - Physiological, environmental, and ecological dimensions of daily heterothermy. On the left: Daily heterothermy involves reversible drops in body temperature below homeothermic levels, typically during rest. Centre: It is triggered by environmental factors such as temperature variability, cold, and food scarcity, which impose energetic constraints. Right: Species expressing daily heterothermy typically inhabit habitats with low productivity and high thermal variability, where homeothermy becomes energetically unsustainable.

What is not known is how daily heterothermy's expression varies across species and environments, and the degree to which it is influenced by ecological variables like ambient temperature and resource availability. Importantly, torpor is not a pathological state, rather it is an actively regulated physiological strategy modulated often by ambient temperature and food availability (Bozinovic *et al.* 2005, Körtner & Geiser 2000). For instance, Chilean mouse-opossums (*Thylamys elegans*) exhibit torpor only under food deprivation, while sugar gliders (*Petaurus breviceps*) adjusted torpor expression to several environmental cues (Bozinovic *et al.* 2005, Körtner & Geiser 2000). Daily heterothermy is taxonomically widespread, occurring among diverse mammalian and avian clades, as well as ecological contexts. However, its distribution, is not as random, as comparative analyses have shown that environmental variables, such as primary productivity and ambient temperature variation are strong predictors of minimum body temperature, which can be used as a proxy for heterothermy level (Nowack *et al.* 2020, Bastos *et al.* 2021). Species expressing daily heterothermy typically inhabit regions with low average ambient temperatures, high thermal variability, and limited primary habitat productivity, conditions where homeothermy demanding physiology becomes energetically unsustainable (Brown & Bartholomew 1969, Körtner & Geiser 2000, Nespolo *et al.* 2010). Experimental data reinforces the environmental sensitivity of daily heterothermy, i.e. in lesser hedgehog tenrec, (*Echinops telfairi*), for which daily torpor bouts were significantly deeper and longer under fluctuating temperatures, when compared to constant conditions (Dausmann *et al.* 2020). Similarly, the wood mouse (*Apodemus flavicollis*) in Central Europe exhibits enhanced heterothermic responses during colder days (Boratyński *et al.* 2018). These empirical results support the hypothesis that heterothermic strategies are shaped by both resource availability and temperature variability (Lovegrove 2000a, Hetem *et al.* 2016, Dammhahn *et al.* 2017). Daily heterothermy constitutes a dynamic thermoregulatory strategy, evolved to optimize energy expenditure under variable environmental conditions. Its continuum of expression, by varying in depth, duration, and frequency both within and across species highlights its role as an evolved response to ecological pressures. Comparative findings underline this, revealing that low minimum body temperatures are most prevalent in species occupying habitats with low productivity and high thermal variability (Nowack *et al.* 2020). Taken together, these views position daily heterothermy as a pivotal trait in the adaptive repertoire of small mammals, particularly under the ongoing climate and habitat changes (Geiser & Turbill 2009, Lovegrove 2017).

1.3. The mechanisms: Molecular and evolutionary basis

Heterothermy is a recognised genetically encoded and ecologically advantageous thermoregulatory strategy that has evolved convergently across vertebrate lineages, underpinned by conserved components (Geiser & Ruf, 1995; Ruf & Geiser, 2015; Lovegrove, 2017; Grigg *et al.*, 2022). It encompasses a continuum of metabolic flexibility that includes daily torpor, seasonal hibernation and transient thermal downregulation, and is thought to have evolved independently across multiple vertebrate lineages (Ruf & Geiser, 2015; Lovegrove, 2017; Grigg *et al.*, 2022). Despite its widespread occurrence, the molecular and evolutionary mechanisms that underlie this physiological flexibility remain insufficiently understood, particularly regarding the extent of convergence versus

lineage-specific innovation. While the diversity of thermoregulatory strategies in mammals from strict homeothermy to daily torpor and hibernation is well documented, the genetic architecture supporting these strategies remains largely unresolved (Carey *et al.*, 2003; van Breukelen & Martin, 2015; Faherty *et al.*, 2018). Early evidence from transcriptomic and comparative genomic studies suggests that thermoregulatory phenotypes are shaped by both conserved modules, such as those controlling mitochondrial function and thyroid signalling, and lineage-specific adaptations involving energy metabolism, circadian regulation, and neuroendocrine pathways (Figure 3; Andrews, 2007; Nowack *et al.*, 2020; Gautier *et al.*, 2018; Crippen *et al.*, 2024).

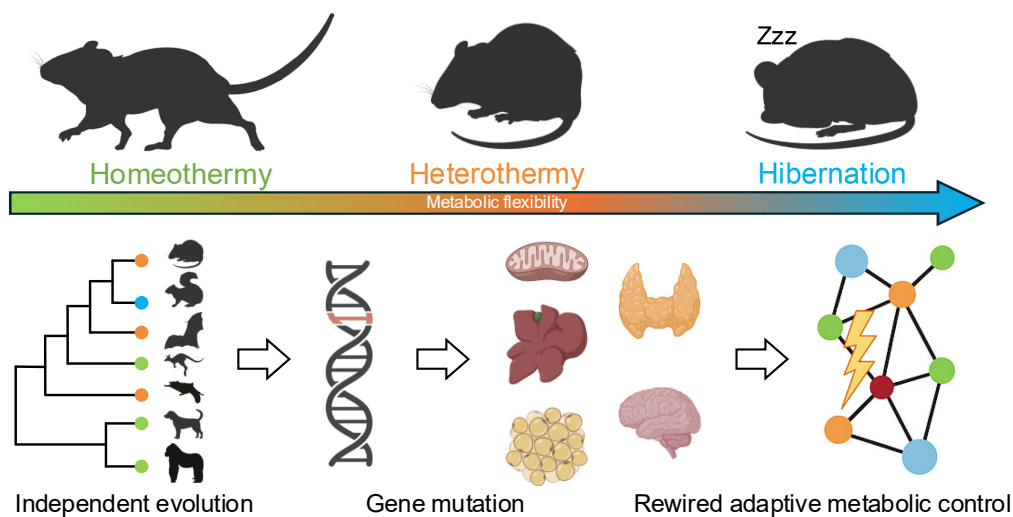


Figure 3 - Evolutionary and molecular architecture of mammalian thermoregulatory strategies. This figure illustrates the continuum from homeothermy (green) to heterothermy (orange) and hibernation (blue), reflecting increasing metabolic flexibility (top panel). Bottom panels depict phylogenetic reconstruction with colour-coded branch ends, indicating independent evolutionary origins of thermoregulatory phenotypes. The role of key thermoregulatory genes (e.g., *Ucp1* and TRP channels) in modulating function across metabolic tissues such as mitochondria, thyroid, BAT, liver, and brain. And the resulting network-level regulation, where adaptive metabolic control emerges from rewired, polygenic architectures.

Particularly, it is unclear whether heterothermic strategies share conserved molecular pathways or whether they have evolved independently across lineages (Carey *et al.*, 2003; Lovegrove, 2017; Grigg *et al.*, 2022). Although phenotypic plasticity in thermoregulation has been broadly recognised (Lovegrove 2017; Bastos *et al.* 2021), the extent to which this plasticity reflects selection on underlying thermoregulatory genes is underexplored. Studies in hibernators and daily heterotherms have suggested that thermoregulatory control involves polygenic traits acting through complex gene regulatory networks (Gautier *et al.* 2018; Crippen *et al.* 2024). Genomic analyses have identified roles for mitochondrial, thyroid, and neuroendocrine genes, though comparative assessments of their conservation and divergence across thermoregulatory phenotypes remain limited (Wu *et al.*, 2015; Zhao *et al.*, 2017; Faherty *et al.*, 2018). It has been proposed that thermoregulation operates as a network-level trait shaped by evolutionary pressures, which is however still to be confirmed by broad comparative studies. The contribution of protein network integration, adaptive amino acid changes, and structural flexibility in core thermogenic pathways remains a critical area of research

(Carey *et al.*, 2003; Andrews, 2019; Tsukamoto *et al.*, 2019). It is still unclear whether these adaptations represent instances of evolutionary convergence or lineage-specific innovations. While signatures of positive selection have been observed in thermoregulatory genes, the consistency and functional implications of these patterns remain insufficiently resolved (Ashkenazy *et al.*, 2016). Some evidence suggests overlapping molecular responses among thermoregulatory phenotypes, yet the extent of convergence is still debated (Wu *et al.*, 2015; Cubuk *et al.*, 2017). Key thermogenic genes such as *Ucp1* are potential targets of lineage-specific adaptation in small heterothermic mammals (Gautier *et al.* 2018). Functional predictions based on structural modelling suggest a possible role in enhancing thermogenic responsiveness, though empirical validation remains needed. The broader significance of these modifications for adaptive thermogenesis remains a subject of active research. Structural variation in thermoregulatory proteins may influence efficiency under variable thermal conditions, but functional studies are limited (Faherty *et al.*, 2016; Romanovsky, 2018). Non-shivering thermogenesis, especially via *Ucp1* in brown adipose tissue, plays a central role in temperature regulation and arousal from torpor (Palkar *et al.* 2015; Andrews 2007). In addition to *Ucp1*, cold-sensitive ion channels and thyroid deiodinases have been implicated in modulating thermal sensitivity and metabolic responses (Romanovsky 2018; Cubuk *et al.* 2017). These molecular systems form part of a complex regulatory architecture supporting diverse thermoregulatory strategies in mammals.

1.4. The benefits: Functional plasticity beyond energy savings

Endotherms exhibit adaptive phenotypic plasticity in response to fluctuating environments, with heterothermy serving as a critical physiological strategy that enhances metabolic flexibility and promotes energy conservation (Ruf *et al.* 2012; Ruf and Geiser 2015; Landes *et al.* 2020). While the energetic benefits of heterothermy are better characterised (Ruf *et al.* 2012; Ruf & Geiser 2015), less is known about how this strategy influences broader biological functions, particularly in relation to brain function and cognitive performance (Figure 4).

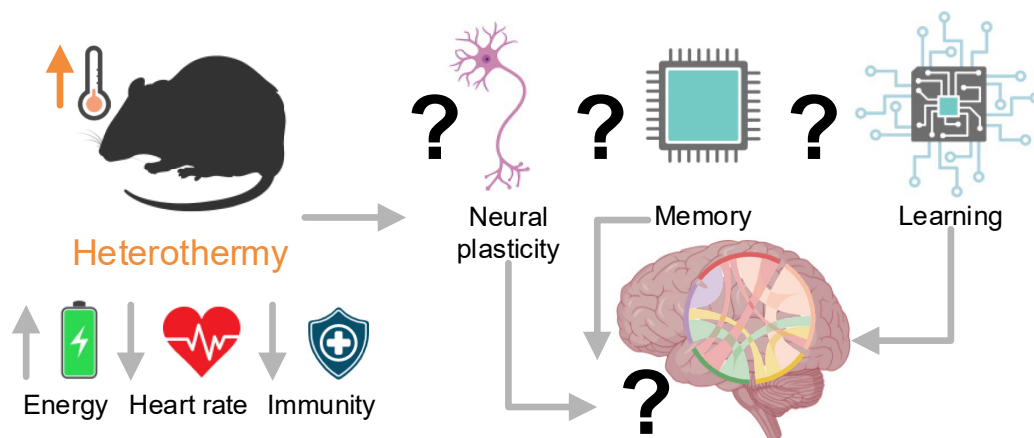


Figure 4 - Potential systemic and cognitive effects of increasing heterothermy in mammals. Greater heterothermy level ($\uparrow$) is associated with energy conservation and physiological suppression (heart rate and immune response). However, its impact on neural plasticity, memory, and learning remains poorly understood. The figure highlights unresolved trade-offs between metabolic efficiency and cognitive performance.

Through cyclical transitions alternating between metabolically active and energy-conserving states such as torpor (IUPS Thermal Commission 2003; Boyles *et al.* 2020), heterothermic species downregulate multiple physiological systems, including metabolism, cardiovascular activity, and immune function, to maintain energetic homeostasis under resource-limited conditions (Carey *et al.* 2003; Klug and Brigham 2015). Increasing attention has been given to the possibility that such flexibility extends beyond energy savings, potentially shaping neurocognitive resilience (Dausmann 2014; Giroud *et al.* 2021). Recent findings suggest that torpor and hibernation may influence neural plasticity, synaptic maintenance, and memory consolidation (Hitrec *et al.* 2019; Jastroch *et al.* 2016), but the precise molecular underpinnings of these effects remain poorly understood. Transitions between metabolic states are associated with significant changes in lipid metabolism, oxidative phosphorylation, and mitochondrial function, with regulation occurring at multiple molecular levels (Carey *et al.* 2003; Ruf *et al.* 2012; Boyles *et al.* 2020). However, how these regulatory mechanisms intersect within gene co-expression networks, particularly those relevant to cognitive functions remains a major unresolved question (Wu *et al.* 2015; Crippen *et al.* 2024). Evidence also suggests that suppression in neural activity during torpor may entail temporary reductions in sensory responsiveness, learning capacity, and threat detection (Nowack *et al.* 2019; Lo Martire *et al.* 2020). These observations have prompted the hypothesis that energetic efficiency gained through heterothermy may come at a cost to neural function, pointing to a possible trade-off that has yet to be mechanistically defined (Jastroch *et al.* 2016).

A key knowledge gap concerns the extent to which heterothermic strategies require and shape transcriptional and network-level plasticity, particularly in species facing extreme thermal variation. Studies in hibernators and daily heterotherms suggest that broader thermal fluctuations may need more dynamic gene regulatory responses during torpor-arousal cycles (Andrews 2007; Crippen *et al.* 2024). However, comparative data across species and tissues remain scarce, especially regarding the involvement of cognitive, neuroendocrine, circadian, and metabolic networks. In small mammals, interspecific variation in heterothermic depth and duration has been reported even among ecologically similar taxa (Bastos *et al.* 2021; Gyhrs *et al.* 2022), yet the molecular correlates of this variation are poorly resolved. Overall, it remains to be determined whether molecular plasticity, particularly at the level of gene co-expression networks, serves as an adaptive mechanism that supports heterothermic expression in diverse ecological contexts (Andrews 2019; Tsukamoto *et al.* 2019). Addressing this gap is critical to understand the full spectrum of physiological trade-offs and advantages associated with heterothermic thermoregulation in mammals.

1.5. The roots: Ontogeny and ancestral thermoregulation signatures

The postnatal development of thermoregulatory capacity remains a comparatively understudied area in thermal biology, despite its fundamental relevance for the understanding of the origin of endothermy in vertebrates. In mammals and birds, the transition from poikilothermic to endothermic states represents a pivotal developmental process, yet its temporal dynamics, regulatory mechanisms, and evolutionary implications are not fully understood. Traditional models propose that this transition

aligns with weaning and the onset of metabolic independence (Koteja 2000; McNab 2002; Koteja 2004), but comparative studies across taxa, particularly in altricial mammals, suggest a protracted and plastic developmental sequence (Koteja 1991; Hayes & Garland 1995). This has led to ongoing debate about whether the acquisition of thermoregulatory autonomy involves a discrete developmental switch or a gradual integration of metabolic, neuroendocrine, and behavioural components (Figure 5).

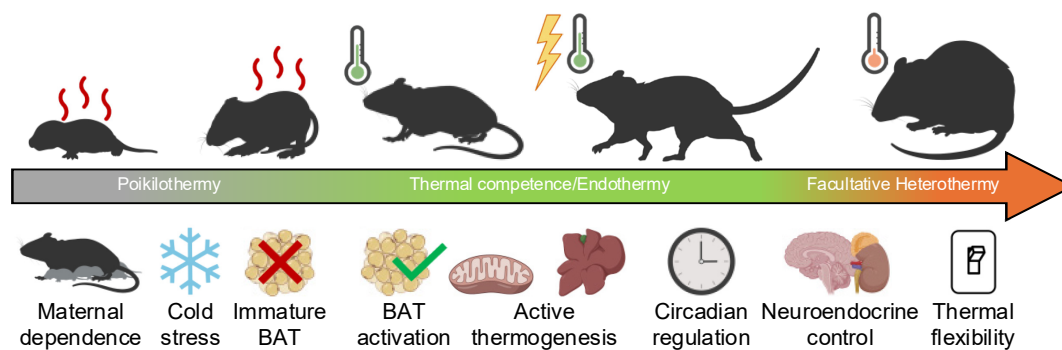
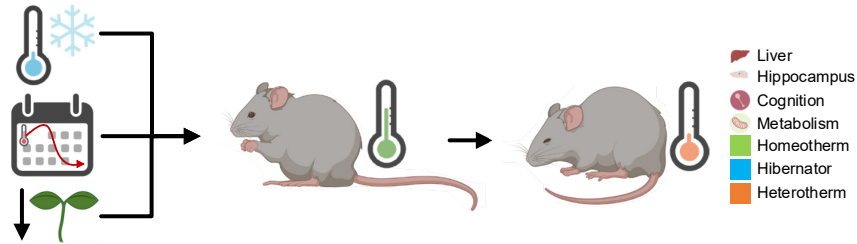


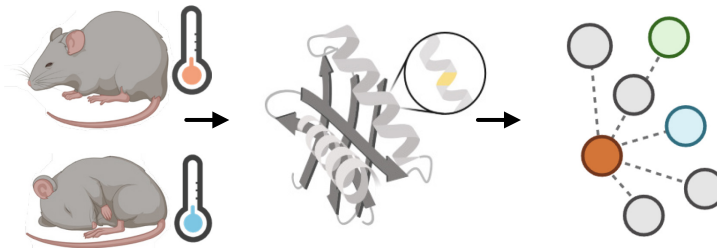
Figure 5 - Postnatal thermoregulatory progression in *Mus musculus*. Ontogeny proceeds from poikilothermic dependence to endothermic competence, involving behavioural (maternal contact), environmental (cold), and physiological (BAT maturation, mitochondrial thermogenesis) mechanisms. In adulthood, facultative heterothermy reflects persistent thermal flexibility regulated by circadian, neuroendocrine, and molecular network inputs.

Current evidence supports the view that thermogenesis and thermal homeostasis emerged along with other developmental milestones, but the timing, sequence, and regulatory control of these transitions varies across taxa (Garland & Carter 1994; Angilletta & Sears 2003; Geiser 2021). Altricial species, such as *Mus musculus*, display a clear ontogenetic trajectory that begins with poikilothermic physiology and progressively increases in thermogenic capacity, driven by both endogenous signals and exogenous cues (Okuda & Koizumi 1989; Blumberg 1997; Zhao *et al.* 2004). This developmental plasticity proposes that early-life thermoregulation is not predetermined but modulated by a combination of hormonal, environmental, and maternal influences. Such flexibility has led some authors to suggest that ontogenetic thermoregulation may reflect evolutionarily conserved patterns that are recapitulated during early development (Koteja 2000; Geiser 2021). Emerging evidence highlights intermediate thermoregulatory phases that rely on behavioural thermo-effectors (e.g., huddling), brown adipose tissue recruitment, and progressive metabolic engagement, rather than a binary switch to homeothermy (Nagy 1993; Cannon & Nedergaard 2004). This nuanced view challenges classical dichotomies and instead supports a continuum model of thermoregulatory evolution, in which poikilothermy and endothermy exist along a flexible spectrum (Geiser & Ruf 1995; Lovegrove 2017). Importantly, facultative heterothermy observed in adult laboratory mice, often presumed strictly homeothermic, underlines the persistence of physiological flexibility beyond early development (Geiser 2008). This convergence between developmental and adult strategies reinforces the suitability of murine models for investigating the molecular, genetic, and systemic determinants of thermal adaptation in vertebrates (Lagerspetz 1966; Cannon & Nedergaard 2004). Nevertheless, several key knowledge gaps persist. The precise timing and mechanistic triggers underlying the transition from poikilothermy to

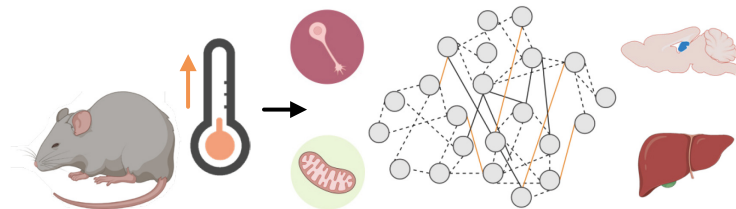
endothermy remain poorly defined, particularly in altricial mammals. Furthermore, the extent to which early-life thermoregulatory plasticity influences long-term physiological adaptability and stress resilience is not well understood. Comparative data is also lacking detailed information about how ontogenetic thermogenic trajectories diverge across taxa and their environmental contexts remains poorly understood, limiting our capacity to generalise developmental patterns across endothermic lineages. Addressing these open questions is essential for advancing a mechanistic and evolutionary understanding of heterothermic thermoregulation in vertebrates (Figure 6).

1- Environmental drivers of minimum body temperature and heterothermy in mammals

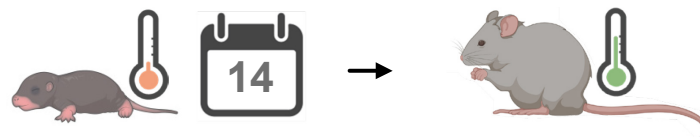
Daily heterothermy increases with lower ambient temperatures, greater among-year temperature variation and low habitat productivity

2- Molecular flexibility and thermoregulatory adaptation in daily heterothermic rodents

Flexible thermoregulating rodents share thermoregulation genes under positive selection, with adaptations integrated into broader protein networks that support complex temperature regulation

3- Co-expression of cognitive and metabolic network pathways across heterothermic levels in desert rodents

Greater daily heterothermy is linked to flexible gene networks in metabolism and cognition, prompting adaptive plasticity under thermal stress.

4- Ontogenic onset of endothermy in laboratory mouse pups in a controlled environment

The Poikilothermy to endothermy shift occurs in mice before weaning at day 14

Figure 6- Thesis chapters and main conclusions overview. Orange codes for heterothermy/poikilothermy, blue for hibernation and green for homeothermy. In point 1, the cold thermometer next to the snowflake means cold ambient temperatures, the calendar with temperature oscillations, means among-year temperature variation and the plant means habitat productivity.

Chapter 2. Objectives and thesis structure

2.1. General objectives

The main aim of this thesis is to investigate the ecological, molecular, regulatory, and developmental determinants of heterothermy in mammals, with an emphasis on the physiological and genetic flexibility that underpins thermal adaptation. This is achieved by integrating large-scale comparative analyses, evolutionary genomics, gene co-expression network profiling, and experimental developmental physiology across a range of mammalian species, from desert rodents to laboratory mice. Specifically, the work explores how environmental variability shapes heterothermic strategies, how molecular signatures of thermoregulation evolve across rodents with different thermoregulatory strategies, how network organization in metabolism and cognition differs across desert species with contrasting heterothermy levels, and how endothermy emerges during postnatal development in laboratory mice.

2.2. Thesis outline

This dissertation is divided into seven chapters, as follows:

Chapter 1 provides an integrative review of the physiological, ecological, molecular, and developmental foundations of thermoregulation in mammals. It introduces core concepts such as endothermy and heterothermy, examines the evolutionary significance of thermal strategies under energetic and climatic constraints, and contextualises the role of molecular, environmental, cognitive, and ontogenetic mechanisms in shaping thermoregulatory flexibility. Chapter 2 presents the main objectives of this dissertation and outlines the structure and rationale behind the research chapters. Chapter 3 investigates the influence of environmental conditions, particularly temperature and productivity, on the minimum body temperature of heterothermic mammals. Using comparative ecological data, the study tests whether interspecific variation in daily heterothermy can be predicted by thermal variability and resource availability across habitats. Chapter 4 examines the molecular evolution of thermoregulation in rodents through the lens of gene sequence variation, protein structure modelling, and protein interaction network integration. It identifies molecular signatures of adaptation in thermoregulation-related genes and evaluates whether species with more flexible thermoregulation exhibit greater evidence of positive selection and protein-level flexibility. Chapter 5 explores how daily heterothermy affects gene co-expression networks regulating metabolism and cognition in desert rodents. The chapter focuses on network size, connectivity, and plasticity across different thermoregulatory phenotypes, offering insights into trade-offs between stability and adaptability in gene regulatory systems. Chapter 6 focuses on the ontogeny of endothermy in laboratory mice. Through a developmental approach, it assesses when and how pups transition from poikilothermy to endothermy, and examines the role of maternal temperature, sex, and nest presence in shaping thermoregulatory capacity. Chapter 7 provides an integrated synthesis of the thesis findings, highlighting the multi-level nature of thermoregulation in mammals. It

consolidates major conclusions from ecological, molecular, network-level, and developmental perspectives, and discusses their implications for understanding thermal adaptation as a continuum rather than a dichotomy. Finally, it outlines future research directions, particularly in the context of climate change, phenotypic plasticity, and thermal ecology.

2.3. Declaration on authorship and transparency in reuse of adapted published material

This dissertation includes original research conducted by the author, as well as content that has been previously published or submitted for publication. Specifically, Chapter 3 incorporates findings from a peer-reviewed article authored by the candidate and co-authors, published in the *Journal of Vertebrate Biology* (Bastos *et al.*, 2021). The inclusion and adaptation of this material has been explicitly approved by all co-authors. All other references to published work throughout the dissertation are fully cited and were not reused in full or adapted in a similar form. No content from publications by other authors has been incorporated beyond standard academic citation. Bibliographic references and authorship contributions are acknowledged in the References chapter, in accordance with the ethical standards of the Faculty of Sciences of the University of Porto.

Chapter 3. Environmental drivers of minimum body temperature and heterothermy in mammals

3.1. Introduction

Precise physiological control of internal body temperature is fundamental to the biology of both birds and mammals (McNab 1978, Lovegrove 2017, Rezende *et al.* 2020). Maintaining a high and stable internal temperature through sustained physiological effort, despite external fluctuations, a process called endothermic homeothermy, has likely been crucial to the ecological and evolutionary success of these groups (Nespolo *et al.* 2011). Nevertheless, a variety of mammals, and to a lesser extent birds, can shortly suspend this strict thermal regulation and body temperature, typically maintained above 30°C (Ruf & Geiser 2015, Nowack *et al.* 2020). This phenomenon, known as daily heterothermy, involves a regulated decrease in body temperature and can provide a selective advantage when the energetic costs of maintaining endothermic homeothermy exceed available resources (Hetem *et al.* 2016, Dammhahn *et al.* 2017, Lovegrove 2017).

The consistently high body temperature maintained by endothermic homeotherms offers various physiological advantages, such as optimized enzymatic activity, the development of complex neural systems, and increased reproductive rates (Nespolo *et al.* 2011, Preußner *et al.* 2017). However, this strategy is metabolically demanding, for instance, an endothermic mammal requires roughly five times the food intake of a similarly sized ectothermic tetrapod (Pough 1980, McNab 2009) just to maintain essential life functions. One way to reduce these energetic costs is through temporal metabolic down-regulation, which leads to variation in body temperature within individuals, as observed in species that hibernate or use daily torpor (Hetem *et al.* 2016, Dammhahn *et al.* 2017, Lovegrove 2017). The ability to suppress metabolism, which is a key feature of heterothermy, is closely tied to energy conservation (Lovegrove 2000a). Advantages of heterothermic strategies include minimized use of energy and water during periods of scarcity (Tieleman & Williams 1999, Vuarin & Henry 2014), and this may explain why facultative heterothermy has evolved multiple times independently in mammals (Geiser 2008). It has even been proposed that heterothermy enabled certain mammalian lineages to survive mass extinction events such as the one that marked the Mesozoic-Cenozoic boundary (Lovegrove 2017), and that heterothermic mammals may continue to face a reduced extinction risk today (Geiser & Turbill 2009). Thus, adopting a heterothermic lifestyle may offer an adaptive advantage in unpredictable or highly variable environments (Geiser & Brigham 2012, Dammhahn *et al.* 2017). Nonetheless, the specific ways in which environmental variation influences heterothermy remain uncertain and under investigation (Vuarin & Henry 2014, Levesque *et al.* 2016, Dammhahn *et al.* 2017).

Heterothermy, defined as intra-individual variation in body temperature, represents a physiological strategy by which animals can adjust internal temperature in response to changes in ambient conditions and resource availability (Bligh & Johnson 1973, Boyles *et al.* 2011, Geiser & Brigham 2012, Vuarin & Henry 2014). However, the simultaneous influence of multiple environmental factors on the extent of heterothermy is still poorly understood (Vuarin & Henry 2014, Dammhahn *et al.* 2017). In hibernating species, for instance, a drop in environmental temperature has been shown to reduce metabolic rates (Geiser 2004, Heldmaier *et al.* 2004), leading to corresponding decreases in both body temperature and total energy expenditure (Geiser 1988). Still, empirical support for a direct link between environmental temperature and body temperature regulation in daily heterotherms is limited (Nespolo *et al.* 2010). Some studies do suggest that declining ambient temperatures can intensify torpor, in particular bird and mammal species (Nowack *et al.* 2017, 2020). For example, in the lesser hedgehog tenrec (*Echinops telfairi*), torpor duration increased, and minimum skin temperature dropped more under variable than constant thermal conditions (Dausmann *et al.* 2020). In wild wood mice (*Apodemus flavicollis*), body temperature variation during winter was inversely related to minimum daily environmental temperatures (Boratyński *et al.* 2018). However, data also indicate that environmental temperature can interact with habitat productivity to shape heterothermic responses, complicating the expected patterns (Nespolo *et al.* 2010).

Experimental studies have shown that even in the absence of low temperatures, food scarcity alone can induce heterothermic responses. For instance, Chilean mouse-opossums (*Thylamys elegans*) exhibited torpor and lower body temperatures only when food was withheld (Bozinovic *et al.* 2005). Similarly, pale kangaroo mice (*Microdipodops pallidus*) failed to enter torpor when overfed but showed increased torpor when both food was limited, and temperatures were low (Brown & Bartholomew 1969). In sugar gliders (*Petaurus breviceps*), the likelihood of entering torpor appeared to reflect ambient temperature, food availability, and thermoregulatory demands influenced by weather patterns (Körtner & Geiser 2000). These findings support the view that a fluctuating environmental temperature, combined with habitat productivity can promote the evolution of daily heterothermy in otherwise homeothermic mammals. Still, the extent to which both temperature and productivity predict interspecific variation in daily heterothermy remains untested.

This following work aims to quantify the relative influence of thermal conditions and environmental energy constraints, specifically those related to habitat productivity, on daily minimum body temperature in mammals that exhibit heterothermy. We propose that variation in ambient temperature is a key determinant of heterothermy levels (Brown & Bartholomew 1969, Körtner & Geiser 2000). Lower average temperatures and higher diurnal temperature range or temperature seasonality may promote temporary reductions in body temperature, thereby increasing heterothermy. We also hypothesize that productivity-related constraints, namely, lower yearly productivity and greater productivity seasonality or among-years productivity variation, can drive the use of heterothermy as a strategy to cope with energetic shortfalls. Furthermore, high among years temperature variation and fluctuations in food availability across years may select for heterothermic responses as an adaptive strategy for dealing with environmental

unpredictability. To evaluate these hypotheses, we used comparative data from mammalian species documented to engage in daily heterothermy (Ruf & Geiser 2015, Nowack *et al.* 2020). We investigated how variation in environmental temperature and habitat productivity predicts interspecific differences in minimum body temperature in several species. We expect species adapted to daily heterothermy to inhabit regions with low yearly temperature and productivity, as well as high variability in these factors. Given that arid environments pose constraints on both food and water, and that many physiological traits scale with body mass, we also examined how body size and adaptation to aridity influence the relationship between minimum body temperature and environmental variables.

Hypothesis:

Environmental variation in thermal conditions and resource availability predicts interspecific differences in the expression of daily heterothermy among mammals.

Research questions:

- Q1 - How does average environmental temperature and its variability (diurnal, seasonal, and among-years) influence the minimum body temperature across mammalian species?
- Q2 - How do levels and seasonal/among-years variability in habitat primary productivity affect minimum body temperature among species?
- Q3 - Does the effect of environmental temperature and productivity on minimum body temperature vary between species adapted to arid versus humid climates?
- Q4 - To what extent does body mass influence or interact with the relationship between environmental variables and minimum body temperature?
- Q5 - Do temperature and productivity predictors act independently or interactively in shaping daily heterothermy?

3.2. Materials and methods

Comparative data

Data on minimum core (internal) body temperature (BT_{min}, °C) and body mass (g) for mammals was gathered from recent reviews (Ruf & Geiser 2015, Nowack *et al.* 2020). Environmental variables were extracted based on the geographic distributions of terrestrial mammals, using Digital Distribution Maps (IUCN 2020). Climate data, including environmental temperature variables (BIO1, BIO2, and BIO4), was sourced from a historical climate repository (Fick & Hijmans 2017) at a resolution of 2.5 minutes and downloaded on 30 October 2020. Range polygons for each species were used to clip the variable rasters, allowing calculation of the median and standard deviation for each species. For analyses, environmental temperature variables included annual median temperature (TM), standard deviation of annual temperature (TSD, representing among-year variation), median diurnal temperature range (TR), and temperature seasonality (TS). The normalized difference vegetation index (NDVI), which represents vegetation density (calculated as the difference between near-infrared and red reflectance divided

by their sum), was derived for the period 1982-2015 (National Center for Atmospheric Research Staff 2018, <https://staff.ucar.edu/browse/orgs/NCAR>, downloaded on 13 November, 2020). Bimonthly NDVI measurements were used to calculate yearly averages and standard deviations of primary productivity. To summarize NDVI data, the mean and standard deviation of average primary productivity across years, as well as the mean of within-year standard deviations, were calculated (Fig. S1). Three indices were derived from summarized NDVI data and species ranges: annual primary productivity (PM, median annual productivity), among-year variation in productivity (PSD, median annual productivity standard deviation), and productivity seasonality (PS, median within-year standard deviations). Based on aridity index data, species were classified as inhabiting arid environments (average aridity index < 0.5) or humid environments (average aridity index ≥ 0.5) (Trabucco & Zomer 2019).

Outlier records were excluded from analyses: five species for BTmin (*Elephantulus rozeti*, *Microcebus berthae*, *Microcebus myoxinus*, *Tarsipes rostratus*, and *Vespadelus vulturnus*) and two species for TM (*Apodemus peninsulae* and *Phodopus sungorus*). This resulted in complete data for 81 mammalian species. A trimmed mammalian phylogeny, including topology and branch lengths (<http://vertlife.org/phylosubsets/>; Upham *et al.* 2019), was applied for comparative analyses.

Statistical analyses

All species and environmental variables were log₁₀-transformed, centred, and scaled. This was done to improve the normality of residual variances and ensure variations were placed on a comparable scale. Since environmental variables showed correlations (Table S1), a principal component analysis (PCA) was performed to address multicollinearity among seven environmental predictors: TM, TSD, TR, TS, PM, PSD, and PS. Principal components were extracted using varimax rotation, with criteria for inclusion being a proportion of explained variance greater than 0.05 and eigenvalues exceeding 1, where the “principal” implemented in “psych” R package was used. These calculated principal components were then used in subsequent analyses. To assess differences in studied variables between arid- and humid-adapted species (Figure 7), ordinary least squares (OLS) and phylogenetic generalized least squares (PGLS) regression analyses were applied. Relationships between BTmin and the principal components were analysed using PGLS, implemented through the “phylolm” R package (Rezende & Diniz-Filho 2012). The influence of principal components on BTmin was evaluated, incorporating body mass as a covariate. A binomial factor for aridity adaptation was also included to distinguish between arid- and humid-adapted species. To investigate whether species size and aridity adaptation influenced responses to environmental predictors, two-way interactions were tested between environmental predictors, the aridity factor, and body mass. Model goodness-of-fit was assessed using the Akaike Information Criterion (AIC) and alternative phylogenetic regression R-squared values for PGLS analyses (Ives 2019). A backward stepwise selection method was applied to the full model, which included all the terms listed above. The model was progressively simplified by removing the least significant interaction terms.

3.3. Results

General patterns of environmental and biological variation

Environmental predictors, derived from the distribution ranges of 81 mammalian species (Figure 7), exhibited pairwise correlations (Pearson's product-moment correlation; $|r|$: 0.05–0.76; and demonstrated distinct variations between species adapted to arid versus humid climates Figure 7, Figure 8, Table 1, Table 2 and Table 3).

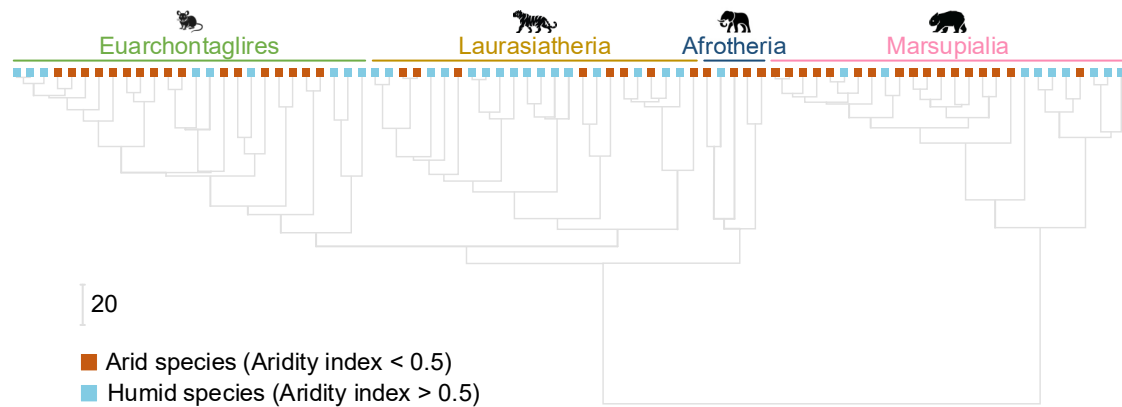


Figure 7 - Phylogenetic relationships among the 81 mammalian species analysed were based on the framework provided by Upham *et al.* (2019).

Specifically, arid-adapted species were associated with environments exhibiting greater diurnal temperature ranges and lower annual productivity, while humid-adapted species tended to occupy more thermally stable regions with higher and more seasonal primary productivity. To quantitatively capture these environmental dimensions, we conducted a principal component analysis (PCA) on seven ecological variables summarising temperature and productivity. This resulted in five rotated components (RCs), explaining 97% of total variance (Table 3). Despite these environmental distinctions, neither minimum body temperature (mean = 18.7 °C; median = 17.9 °C; range = 7.7–31.0 °C) nor body mass (mean = 335.4 g; median = 27.0 g; range = 2.0–9000.0 g) showed statistically significant differences between arid and humid species groups ($P > 0.25$; Figure 3). BTmin and body mass distributions were heavily skewed towards small-bodied species, yet spanned over three orders of magnitude, providing a robust cross-taxonomic perspective on thermoregulatory diversity (Table 2). Furthermore, environmental predictors were only moderately correlated with minimum body temperature (BTmin) ($|r| < 0.38$, $P > 0.001$) and weakly associated with body mass ($|r| < 0.17$, $P > 0.15$; Table S1).

Notably, a simple phylogenetic univariate regression revealed a weak but significant positive relationship between body mass and BTmin ($\beta_{pGLS} = 0.28 \pm 0.13$ SE; $t = 2.16$, $P = 0.034$), although this effect did not persist in multivariate models incorporating environmental covariates. This suggests that body mass may weakly co-vary with

thermal traits and is not a major driver of thermoregulatory strategy across species when environmental context is accounted for. These findings highlight the complexity of disentangling environmental and biological contributions to thermoregulatory traits, while supporting the hypothesis that environmental temperature variation and primary productivity jointly shape patterns of thermal adaptation across mammals (Bastos *et al.*, 2021).

Table 1 - The correlation matrix of variables for the 81 mammalian species examined includes BT_{min} (minimum core body temperature) and BM (body mass). Bioclimatic variables analysed were the annual median (T_M) and standard deviation (T_{SD}) of environmental temperature (BIO1), the median diurnal temperature range (T_R; BIO2), and temperature seasonality (T_S; BIO4). Indices for primary productivity were derived from NDVI data and species distribution ranges, including the primary productivity (P_M), among years variation in productivity (P_{SD}; median of standard deviations of yearly averages), and intra-annual productivity seasonality (P_S; median of within-years standard deviations). Significance levels are denoted as *p ≤ 0.05 and **p ≤ 0.001.

	BT _{min}	BM	T _M	T _{SD}	T _R	T _S	P _M	P _S
BM	0.22*	-	-	-	-	-	-	-
T _M	0.14	-0.16	-	-	-	-	-	-
T _{SD}	0.01	-0.15	-0.37**	-	-	-	-	-
T _R	-0.23*	0.02	-0.05	-0.20	-	-	-	-
T _S	-0.36**	0.02	-0.64**	0.06	0.59**	-	-	-
P _M	0.38**	0.01	0.19	0.18	-0.76**	-0.64**	-	-
P _S	0.37**	0.13	-0.27*	0.28**	-0.36**	-0.14	0.68**	-
P _{SD}	0.23*	0.09	0.11	-0.09	-0.04	0.04	0.36**	0.57**

Table 2 - Differences in the analysed variables between species from arid (factor level = 1) and non-arid species (level = 0). The results are presented for ordinary least squares (OLS) and phylogenetic generalized least squares (PGLR) models

variable	Humid species (AI≥0.5)			Arid species (AI<0.5)			OLS			PGLS			
	mean (S.D)	min- max	me d	mean (S.D)	min- max	me d	β(S.E)	t	p	βPGLS (S.E)	t	p	λ
Minimum body temperature (°C)	19.4 (5.1)	10.4- 28.6	18. 6	18.2 (5.9)	7.7- 31	17. 1	-0.25 (0.22)	$\bar{1.1}$ 4	0.26	-0.08 (0.23)	$\bar{0.3}$ 3	0.74	0.8 53
Body mass (g)	160.9 (506.0)	2-2880	28	468.3 (1847.2)	2593 314	468 .3	0.07 (0.23)	0.2 9	0.77	-0.02 (0.16)	$\bar{0.1}$ 3	0.89	1
Yearly temperature (°C)	19.9 (6.7)	4.5- 26.8	22. 7	18.4 (5.2)	5.6- 26.2	18. 4	-0.12 (0.25)	$\bar{0.5}$ 5	0.59	0.18 (0.22)	0.8	0.42	0.7 23
Among years t. variation (°C)	3.5 (2.1)	0.4- 11.0	2.8	2.6 (1.4)	0.3- 6.8	2.2	-0.49 (0.22)	$\bar{2.2}$ 6	0.02 7	-0.38 (0.22)	$\bar{1.7}$ 3	0.08 8	0.4 08
Diurnal temperature range (°C)	11.2 (1.6)	8.4- 13.9	11. 2	14.4 (1.3)	11.2- 16.5	14. 6	1.48 (0.15)	9.7 4	<.0 001	1.27 (0.15)	8.4 1	<.0 001	0.8 5
Temperature seasonality (°C)	3.1 (2.9)	0.5-9.7	2.4	5.3 (2.0)	1.0- 10.7	5.1	1.05 (0.19)	5.4 6	<.0 001	0.35 (0.15)	2.3 6	0.02 1	0.9 67
Yearly productivity	0.63 (0.13)	0.39- 0.84	0.6 3	0.30 (0.11)	0.14- 0.50	0.2 8	-1.59 (0.14)	$\bar{11.}$ 5	<.0 001	-1.59 (0.14)	$\bar{11.}$ 5	<.0 001	<.0 001
Productivity seasonality	0.09 (0.03)	0.05- 0.18	0.0 8	0.06 (0.03)	0.01- 0.13	0.0 6	-0.93 (0.20)	$\bar{4.6}$ 4	<.0 001	-0.89 (0.20)	$\bar{4.4}$ 1	<.0 001	0.1 59
Among-years p. variation	0.02 (0.003)	0.02- 0.03	0.0 2	0.02 (0.006)	0.01- 0.04	0.0 2	-0.10 (0.22)	$\bar{0.4}$ 6	0.65	-0.10 (0.22)	$\bar{0.4}$ 6	0.65	<.0 001

Despite these environmental contrasts, neither body temperature (mean = 18.7, median = 17.9, range = 7.7-31.0 °C) nor body mass (mean = 335.4, median = 27.0, range = 2.0-9000.0 g) differed significantly between arid and humid species groups ($P > 0.25$; Figure 8). Distributions of BTmin and body mass were skewed towards small-bodied species but spanned more than three orders of magnitude, providing a robust cross-taxonomic perspective on thermoregulatory diversity. Environmental predictors showed only moderate correlations with BTmin ($|r| < 0.38$, $P > 0.001$) and weak associations with body mass ($|r| < 0.17$, $P > 0.15$; Table S1), suggesting that no single environmental variable dominated the observed patterns

A univariate phylogenetic regression revealed a weak but significant positive association between body mass and BTmin ($\beta_{\text{PGLS}} = 0.28 \pm 0.13 \text{ SE}$; $t = 2.16$, $P = 0.034$); however,

this effect was no longer significant when environmental variables were included in multivariate models, suggesting that body mass alone does not explain interspecific variation in thermoregulatory strategies.

Table 3- Results of the rotated principal component analysis of environmental predictors derived from the distribution ranges of 81 mammalian species. The table shows the percentages variation in environmental variables explained by the rotated principal components (RCs) and the standardized loadings of these variables to each component. Key environmental predictors are highlighted in bold for each component.

	RC1 (25%)	RC2 (23%)	RC3 (16%)	RC4 (15%)	RC5 (18%)
Yearly temperature	-0.01	0.94	0.14	-0.2	-0.19
Among-years temperature Δ	-0.1	-0.16	-0.06	0.97	0.12
Diurnal temperature range	0.98	-0.09	0.01	-0.1	-0.13
Temperature seasonality	0.51	-0.78	0.18	0.03	-0.24
Yearly productivity	-0.69	0.29	0.21	0.1	0.56
Productivity seasonality	-0.25	-0.15	0.37	0.16	0.85
Among-years productivity Δ	-0.02	0.04	0.95	-0.07	0.27

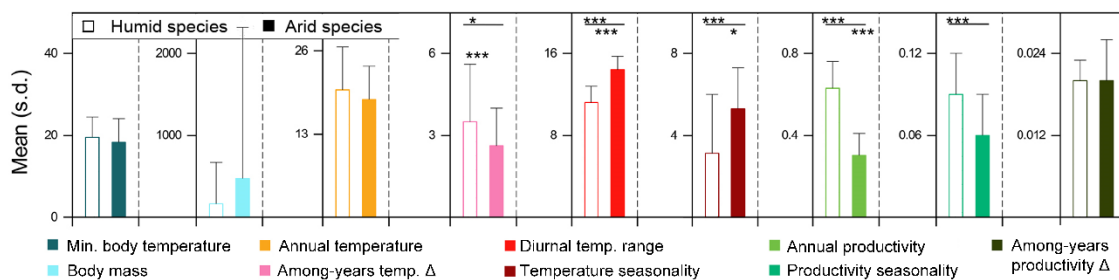


Figure 8 - Mean and standard deviations of the studied variables, along with the differences between species from arid and non-arid climates (Table 2). *P < 0.05 and ***P < 0.0001 represent statistically significant differences, as tested using ordinary (above) and phylogenetic (below) generalized least squares.

Minimum body temperature is lower in cold and thermally variable environments

A total of five rotated principal components accounted for 97% of the variation observed among the species, based on seven environmental predictors derived from their distribution ranges (Figure 8, Table 3). The first component (RC1) was mainly influenced by diurnal temperature range, annual productivity, and temperature seasonality. The second component (RC2) was primarily influenced by annual temperature and temperature seasonality. The third (RC3) and fourth (RC4) components were driven by among-year variation, either in productivity or in temperature, respectively. The fifth component (RC5) was associated with annual productivity and productivity seasonality (Figure 8). Environmental variables significantly explained interspecific variation in

minimum body temperature (BT_{min}) accounting for approximately 50% of the observed variance across the 81 mammalian species (Figure 9, Table 4 and Table 11).

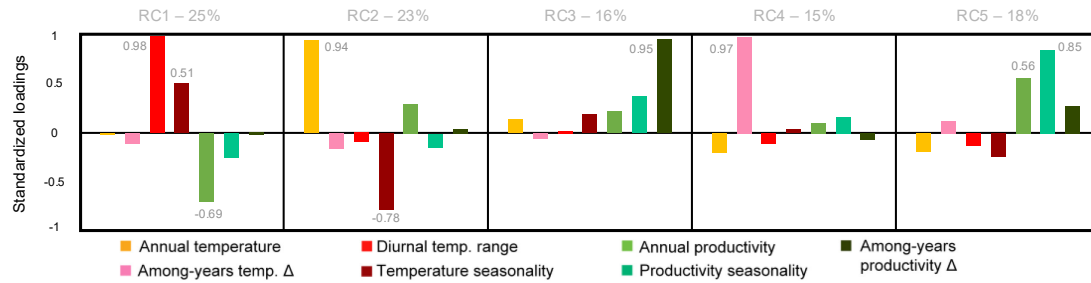


Figure 9 - Overview of the rotated principal component analysis for environmental predictors derived from species ranges of 81 mammals. The table (Table 3) presents the percentages of variation explained by the principal components (RCs) and the standardized loadings (> 0.50) of environmental variable to the components.

Table 4 - Parameter estimates for phylogenetically generalized least squares (PGLS) analysis of environmental variables and body mass in predicting minimum body temperature in mammals ($\lambda = 0.849$, AIC = 207.02, $n = 81$). The "arid factor" refers to species from arid (0; aridity index < 0.5) or humid climates (1; aridity index > 0.5), with RC, representing rotated principal components (Fig. 3). Standard regression, R-squared values in PGLS analyses are uncertain; thus, alternative values are provided: $R_{ls}^2 = 0.52$, $R_{ce}^2 = 0.55$, $R_{lr}^2 = 0.42$ (Ives 2019). The left panel presents results from an analysis that includes the non-significant terms of body mass and RC3, which are excluded (NA) in the analysis in the right panel

	$\beta_{PGLS}(ST)$	t	P	$\beta_{PGLS}(ST)$	t	P
Intercept	-0.34 (0.66)	-0.52	0.61	-0.36 (0.68)	-0.53	0.60
Arid (0,1)	0.38 (0.30)	1.26	0.21	0.45 (0.30)	1.50	0.14
Body mass	0.21 (0.12)	1.70	0.093	NA	NA	NA
RC1	-0.26 (0.15)	-1.71	0.091	-0.33 (0.15)	-2.14	0.035
RC2	0.27 (0.11)	2.37	0.020	0.28 (0.11)	2.47	0.016
RC3	0.13 (0.10)	1.37	0.18	NA	NA	NA
RC4	-0.35 (0.15)	-2.30	0.024	-0.34 (0.16)	-2.19	0.032
RC5	0.38 (0.12)	3.30	0.001	0.47 (0.11)	4.36	<0.0001
Arid*RC4	0.57 (0.18)	3.22	0.002	0.53 (0.18)	2.96	0.0041

Table 5 - Model selection procedure used in the study. The full model incorporated the aridity factor, body mass, five principal components and two-way interactions among the predictors. The term "reduced predictor" refers to a predictor that was initial part of the full model (see: "statistics of reduced predictor") but excluded in the specific analysis here presented

Reduced predictor	Model statistics		Statistics of reduced predictor		
	AIC	Δ AIC	β PGLS (s.e.)	t	p
Full model	220.52	-	-	-	-
Body mass*RC4	218.52	2.00	0.01 (0.12)	0.03	0.97
Body mass*RC2	216.58	1.94	-0.03 (0.12)	-0.21	0.84
Arid*RC5	214.67	1.91	-0.14 (0.54)	-0.27	0.79
Arid*RC2	213.02	1.65	0.12 (0.22)	0.54	0.59
Body mass*RC5	211.50	1.52	0.07 (0.11)	0.64	0.53
Body mass*RC1	210.63	0.87	0.14 (0.14)	0.97	0.33
Body mass*Arid	209.15	1.48	-0.12 (0.18)	-0.67	0.41
Arid*RC1	208.02	1.13	0.23 (0.27)	0.87	0.39
Body mass*RC3	207.6	0.42	0.16 (0.14)	1.18	0.24
Arid*RC3	207.2	0.4	0.29 (0.26)	1.12	0.26

Among-year variation in primary productivity (RC3) did not show a significant correlation with minimum body temperature (BTmin; β PGLS (SE) = 0.13 (0.10), $t = 1.37$, $P = 0.18$) and among species variation in body mass had a weak association with BTmin (β PGLS (SE) = 0.21 (0.12), $t = 1.70$, $P = 0.093$). The negative trend of RC1 on BTmin (β PGLS (SE) = -0.26 (0.15), $t = -1.71$, $P = 0.091$) became significant (β PGLS (SE) = -0.33 (0.15), $t = -2.14$, $P = 0.035$) after the stepwise backward reduction of the insignificant effects of RC3 and body mass (Table 10, Table 4). BTmin was negatively associated with diurnal temperature range and seasonality but positively related to annual productivity (Figure 9). These relationships indicate that low and variable environmental temperatures, particularly diurnal and seasonal fluctuations, independently predict lower minimum body temperatures in mammals (Q1).

Low primary productivity and narrow seasonality drive heterothermy

The positive effects of RC2 (β PGLS (SE) = 0.27 (0.11), $t = 2.37$, $P = 0.020$) and RC5 (β PGLS (SE) = 0.38 (0.12), $t = 3.30$, $P = 0.0015$) indicate positive associations with annual temperature, annual productivity and productivity seasonality, whereas RC1 included variables negatively associated with BTmin, namely diurnal temperature range and temperature seasonality (Figure 9, Figure 10 and Table 6).

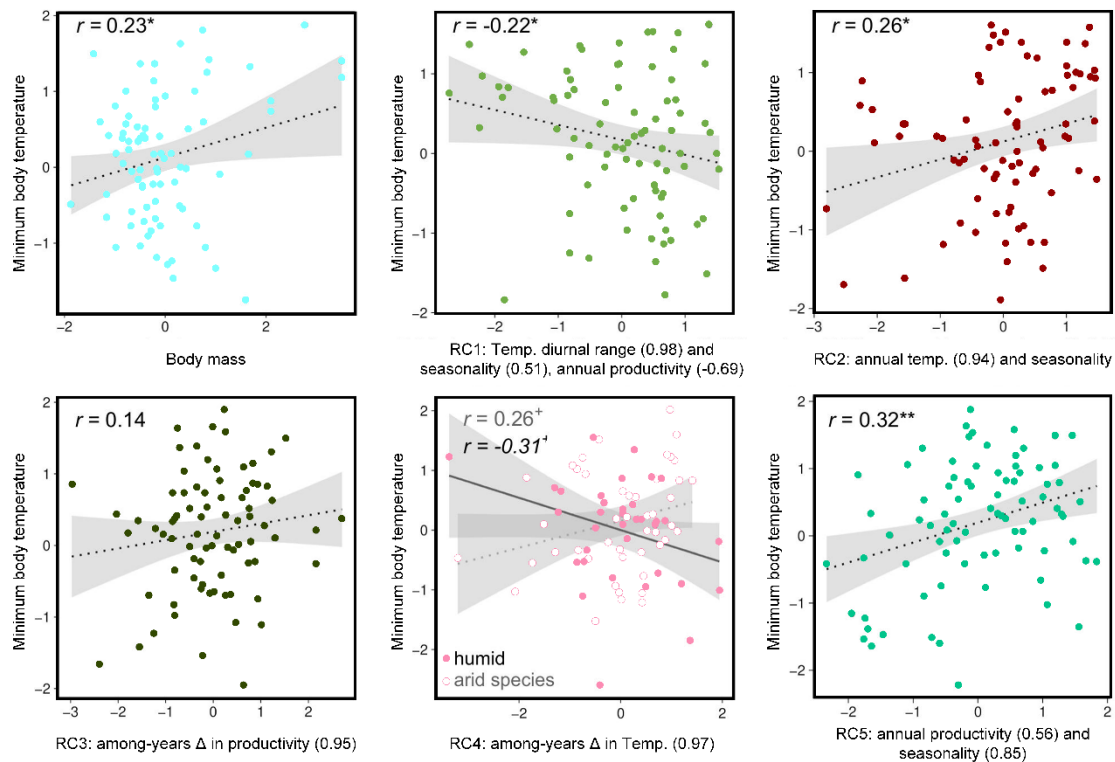


Figure 10 - Bivariate plots illustrating the relationships between traits from the multivariate phylogenetically generalized least squares (PGLS) results. The plots display regression lines ($\pm$ confidence intervals) against raw data points (dots). Residuals of minimum body temperature are shown in relation to body mass and principal components derived from environmental predictors (Figure 2). These residuals were calculated from the PGLS regression of minimum body temperature on predictors in the multivariate analysis (Table 4), excluding the plotted predictor for each panel. Pearson's product-moment correlations (r) are shown, with significance levels indicated ($^{**}P < 0.01$, $^*P < 0.05$, $^+P < 0.08$). The x-axis is logged and includes standardized loadings of environmental variables to the principal components for variables with loadings $> 50\%$ (Figure 3).

Overall, these results suggest that lower levels and seasonality of primary productivity are significantly associated with lower BTmin (Q2), while among-year variability in productivity (RC3) is not a significant predictor. This highlights that not just the amount of productivity, but its seasonality plays a role in shaping thermoregulatory strategies.

Aridity modulates the effect of temperature unpredictability, while body mass has no effects on BTmin

The relationship between minimum body temperature (BTmin) and among-year variation in environmental temperature (RC4) showed different directions for species from arid and humid climates, as revealed by a significant interaction (Arid*RC4: Table 3). Following this, principal component analyses (PCA) and phylogenetically generalized least squares (PGLS) were conducted on datasets focused on species either from arid ($AI < 0.5$) or humid climates ($AI \geq 0.5$). In species from humid climates, minimum body temperature was negatively correlated with among-year variation in environmental temperature ($\beta\text{PGLS (SE)} = -0.42 (0.15)$, $t = -2.83$, $P = 0.0086$, $\lambda = 0.849$, $AIC = 103.4$, $n = 35$). The positive trend in correlation between BTmin and among-year variation in environmental temperature for species from arid climates was not significant ($\beta\text{PGLS (SE)} = 0.13 (0.13)$, $t = 1.05$, $P = 0.30$, $\lambda = 0.614$, $AIC = 123.6$, $n = 46$; Tables 6 and 7). Body mass did not influence any of the identified association, as the interactions between body mass and environmental predictors on BTmin were not significant ($|\beta\text{PGLS}| < 0.16$, $|t| < 1.18$, $P > 0.24$). This finding reveals that the predictive strength of inter-annual temperature variability is context-dependent and moderated by climatic regime.

Table 6 - Results from two rotated principal component analyses of environmental predictors calculated from species ranges of 46 arid and 35 not-arid mammals. The percentages of variation explained by each rotated principal component (RC) are provided, along with the standardized loadings of environmental variables for each given component.

	RC1		RC2		RC3		RC4		RC5	
mammal	arid	humid	arid	humid	arid	humid	arid	humid	arid	humid
%	(35%)	(31%)	(16%)	(18%)	(15%)	(16%)	(15%)	(16%)	(15%)	(16%)
Yearly temperature	-0.03	-0.91	0.89	0.05	0.12	-0.02	-0.20	-0.22	-0.36	-0.26
Among-years temperature variation	0.05	0.24	-0.15	-0.03	0.00	-0.04	0.99	0.96	0.04	0.1
Diurnal temperature range	-0.20	0.1	0.09	0.95	0.96	0.13	0.00	-0.05	0.15	0.23
Temperature seasonality	-0.12	0.86	-0.28	0.25	0.18	0.3	0.03	0.14	0.93	0.17
Yearly productivity	0.89	-0.6	-0.03	-0.41	-0.25	-0.24	0.11	-0.31	-0.21	-0.46
Productivity seasonality	0.91	0.35	-0.33	0.28	-0.11	0.13	0.10	0.11	-0.09	0.87
Among-years productivity variation	0.88	0.18	0.38	0.13	-0.03	0.97	-0.14	-0.04	0.10	0.12

Table 7 - Parameter estimates for two phylogenetically generalized least squares (PGLS) analyses of environmental variables predicting minimum body temperature in arid ($R_{ls}^2 = 0.50$, $R_{ce}^2 = 0.47$, $R_{lr}^2 = 0.41$) and not-arid mammalian species ($R_{ls}^2 = 0.44$, $R_{ce}^2 = 0.44$, $R_{lr}^2 = 0.31$).

	β PGLS (s.e.)		t		p	
	arid	humid	arid	humid	arid	humid
mammal						
Intercept	-0.15 (0.47)	-0.14 (0.69)	-0.31	-0.2	0.76	0.84
IBM	0.33 (0.15)	0.27 (0.20)	2.19	1.35	0.034	0.19
RC1	0.37 (0.12)	-0.21 (0.20)	3.01	-1.06	0.005	0.3
RC2	0.21 (0.15)	-0.13 (0.16)	1.38	-0.84	0.18	0.41
RC3	-0.10 (0.14)	-0.12 (0.15)	-0.76	-0.8	0.45	0.43
RC4	0.13 (0.13)	-0.42 (0.15)	1.05	-2.83	0.3	0.009
RC5	-0.09 (0.13)	0.09 (0.16)	-0.64	0.59	0.52	0.56
arid: $\lambda=0.614$, AIC=123.6, N=46			humid: $\lambda=0.846$, AIC=103.4, N=35			

Overall, our results indicate that the effect of interannual variation in environmental temperature on BTmin is climate-dependent, exerting a stronger influence in humid-adapted species than in arid-adapted ones (Q3). Body mass appears to play a comparatively minor role in shaping minimum body temperature, as no significant interactions were detected between body mass and environmental predictors (Q4). Moreover, temperature and productivity variables independently contribute to variation in BTmin, with no strong evidence for interaction effects between them (Q5).

3.4. Discussion

Physiological regulation of core body temperature plays a critical role in endothermic animals (Ruben 1995, Lovegrove 2017), and is often suggested to have been essential for the ecological and evolutionary success of birds and mammals (Koteja 2000, Lovegrove 2017). Nevertheless, increasing recognition highlights the deviations from strict homeothermic regulation in numerous mammalian lineages, leading to the development of the ability of temporarily abandon homeothermy, such as in the case of daily heterothermy (Geiser 1988, Boyles *et al.* 2011, Geiser & Brigham 2012, Dammhahn *et al.* 2017). The phylogenetic analysis from this study suggest that minimum body temperature can be predicted by environmental factors (Table 4). Together, variation in primary productivity and thermal environment accounted for approximately half of the observed variation in minimum body temperature across the studied species. Specifically, low primary productivity and limited seasonality in productivity were associated with low minimum body temperature observed in certain species. Similarly, low environmental temperature along with greater daily and seasonal variation in temperature, predicted lower minimum body temperature. Interestingly, among-year variation in primary productivity had little impact on the differences in minimum body

temperature between species. In contrast, among-year variation in environmental temperature had contrasting effect on minimum body temperature in mammals from arid versus humid climates.

It was hypothesized that mammals inhabiting warmer climates would exhibit less heterothermy compared to those in colder regions (Nowack *et al.* 2017, Boratyński *et al.* 2018, Dausmann *et al.* 2020). Our findings confirmed these hypotheses, showing that higher annual environmental temperature (the dominant factor in the second principal component: Figure 9) were associated with higher minimum body temperature in the studied mammals (Figure 10, Table 4). Beyond just annual temperature, it was also found that factors influencing temperature variability played a significant role in explaining variation in minimum body temperature. A broad diurnal temperature range (the primary factor in the first principal component: Figure 9) was observed in regions inhabited by mammals with comparatively low minimum body temperatures (Figure 9). Additionally, significant seasonal temperature variation (a major factor in both the first and second principal components) was found in regions inhabited by mammals with lower minimum body temperatures (Figure 9). Thus, it can be inferred that lower annual environmental temperatures and greater daily and seasonal temperature fluctuations are linked to the evolution of mechanisms for temporal down-regulation of body temperature in mammals. Notably, the impacts on annual temperature and daily temperature range independently contributed to the variation observed in minimum body temperature. These environmental factors were strongly correlated (Table 1) and influenced two distinct environmental principal components, both of which are crucial predictors of daily heterothermy in mammals (Figure 9). The statistical independence proposes that changes in these two predictors can independently contribute to the evolution of heterothermy, and when both factors occur together, they may enhance the evolutionary and physiological responses in the species exposed to them. As a result, regions with large daily temperature variations and low annual temperatures, including slightly cold arid and semi-arid areas and high-altitude equatorial regions, are expected to be home to mammals exhibiting daily heterothermy physiology (Geiser & Turbill 2009, McKechnie & Mzilikazi 2011, Okrouhlík *et al.* 2021). The among-year variation in environmental temperature exerts differing impacts on mammals from arid regions compared to those in humid areas (Figure 10, Table 4). In mammals living in more humid environments, this influence was pronounced (Table 6), with species in areas of significant annual temperature variability showing reduced minimum body temperatures (Figure 9). Previous research has proposed that increased level of heterothermy in typically homeothermic animals may enable species to endure extreme climate shifts (Boyles *et al.* 2011, Geiser & Brigham 2012). The robust negative correlation between minimum body temperature and annual temperature variation represents the first quantification of the relationship supporting this hypothesis.

Our comparative findings indicate that endothermic species in regions with higher temperature unpredictability may have evolved temporal suspensions from homeothermy, aiding their survival under extreme and erratic thermal conditions. This adaptation appears absent in species from arid environments, where additional constraints, including limited primary productivity (Table 6 and Table 7) and

environmental temperature variation, restrict both physiological and population-level responses (Lovegrove 2000b, Barros *et al.* 2018).

Resource availability, shaped by fluctuations in habitat productivity, can play a role in influencing daily heterothermy levels (Brown & Bartholomew 1969, Bozinovic *et al.* 2005, Nespolo *et al.* 2010). When environmental resources are insufficient to meet the energy requirements of homeothermic endothermy, low primary productivity may trigger temporary downregulations in body temperature. Our analysis confirmed that low annual environmental productivity (linked to the second and fifth principal components: Figure 9) was associated with low level of minimum body temperatures in certain mammals (Fig. 4, Table 4). Seasonality in primary productivity showed a positive correlation with its annual level (also tied to the fifth principal component: Figure 9), indicating greater seasonal variation in productivity in regions with higher productivity. Both the annual productivity level and seasonality in productivity exhibited the strongest positive correlation with minimum body temperatures across mammals (Table 4). Annual productivity showed a negative correlation with both diurnal temperature range and temperature seasonality, contributing to the first principal component (Figure 9). The combination of low annual productivity and greater temperature variation created environmental conditions that favoured heterothermy in mammals (Figure 10). By comparison, among-year variation in primary productivity was the weakest predictor of the observed differences in minimum body temperatures among the mammalian species studied. Therefore, it can be inferred that a baseline level of primary productivity is essential for sustaining the energetically demanding process of homeothermic endothermy, and when this threshold is unmet in conjunction with wide temperature fluctuations, heterothermy likely evolves.

Even with body masses spanning over three orders of magnitude in our dataset, the environmental influences on daily heterothermy appeared unrelated to the size of the mammals studied. We acknowledge that our findings are based on data from just 81 mammalian species, so they do not provide conclusive evidence for the lack of body mass effects on environmental limitations to daily heterothermy. Nonetheless, if these findings hold true, they are suggestive of environmental factors exerting comparable selective pressures on daily temperature regulation across mammals of varying sizes.

3.5. Conclusion

All animals acquire energy from their surroundings, convert it, and distribute it among physiological processes, which ultimately contribute to their fitness (Boratyński & Koteja 2009, Boratyński *et al.* 2010, Dammhahn *et al.* 2017, Boratyński & Szafrńska 2018, Boratyński 2020, Desforges *et al.* 2020, Pettersen *et al.* 2020). Our study demonstrated that primary productivity and environmental temperature are key ecological predictors determining the minimum body temperature observed in mammals. A downregulation in physiological processes leads to minimum body temperatures, serving as an indicator of the degree of heterothermy exhibited (Brown & Bartholomew 1969, Körtner & Geiser 2000). Consequently, our findings support the idea that daily heterothermy, an energy-saving physiological strategy, is likely to evolve in mammals in response to

environmental variation. Among-year variation in environmental temperature, regardless of changes in habitat productivity, was a strong predictor of low minimum body temperatures in mammalian species. This effect was particularly strong in humid-climate species, which may be more sensitive to inter-annual thermal variability, possibly due to lower thermal adaptation thresholds. This finding aligns with the hypothesis that mammals capable of daily body temperature down-regulation may have a survival advantage against extinction caused by shifts in environmental temperatures (Lovegrove 2000b, Boyles *et al.* 2011). Our results indicate that this mechanism is absent in species adapted to arid environments, highlighting their heightened vulnerability to habitat and climate changes (Brito *et al.* 2016). Additionally, low primary productivity strongly predicted low minimum body temperatures, confirming that heterothermy develops when energy demands for maintaining homeothermic endothermy are unmet (Rezende *et al.* 2020). Overall, our results demonstrate that specific environmental conditions drive the occurrence of low minimum body temperatures. Specifically, species with low minimum body temperatures are predicted to inhabit areas characterized by low yet variable ambient temperatures and limited seasonality in environmental productivity. Future studies should integrate direct physiological recordings of torpor bouts and daily temperature rhythms, ideally paired with measures of energy intake, activity, and metabolic rates, to validate these macroecological associations (Boratyński *et al.* 2020, Nowack *et al.* 2020).

Chapter 4. Molecular network flexibility and thermoregulatory adaptation in daily heterothermic rodents

4.1. Introduction

Thermoregulation is a physiological and behavioural process allowing organisms to adjust their body temperature in response to changing environmental conditions, optimizing cellular and molecular functions (Sealfon and Zorogniotti 1991, Mota-Rojas *et al.* 2021). Endotherms, including mammals and birds, have evolved the ability to maintain high and relatively constant body temperatures, despite environmental fluctuations (Lovegrove, 2017; Bastos *et al.* 2021). The origins of endothermic homeothermy have been a subject of extensive debate (Carey *et al.*, 2003; Koteja 2004; van Breukelen and Martin 2015; Lovegrove, 2017; Grigg *et al.* 2022). While homeothermy offers fitness advantages by optimizing the thermal environment for cellular processes (Hetem *et al.* 2016), research highlights that its evolution may be more complex than previously believed (van Breukelen and Martin, 2015). Endothermic homeothermy comes with high energetic costs (Ruf and Geiser, 2015). Mammals need at least ten times more energy than reptiles of similar sizes (Pough 1980, Gillooly *et al.* 2017). In resource-limited environments, evolution should favour species that minimize energetic costs (Ruf and Geiser, 2015). To cope with energy constraints, some mammals and birds exhibit temporal reductions in metabolic rate and body temperature, allowing them to conserve energy during challenging conditions (Vuarin and Henry, 2014, Bastos *et al.* 2021, Gyhrs *et al.* 2022). Such metabolic flexibility is thought to have emerged early in mammalian evolution, possibly co-evolving with or preceding the full establishment of homeothermic endothermy. Early mammals may have already employed torpor and hibernation to reduce energy expenditure (van Breukelen and Martin, 2015), yet the molecular basis of these strategies remains less studied than that of strict homeothermy (Augee and Gooden 1992; Grigg *et al.* 2004; Angilletta *et al.*, 2010; Lovegrove, 2017). While homeothermy is linked to thermoregulation, cold adaptation, Mesozoic climatic shifts, and the transition from ecto- to endo- thermy (Carey *et al.*, 2003), its evolution was likely influenced by broader ecological pressures, including parental provisioning, increased aerobic capacity and sustained activity (Bennett and Ruben 1979; Farmer 2000).

Thermoregulation mechanisms include shivering and non-shivering thermogenesis, cutaneous vasomotion, sweating, panting and piloerection (Nakamura, 2011). Throughout the body, thermosensitive neurons in the skin and internal organs transfer information on environmental temperature variations to the central nervous system, where it is processed in the preoptic area of the hypothalamus, which is the primary thermoregulatory centre of the brain, with light-dark cycles influencing thermoregulatory adjustments via the pineal gland's secretion of melatonin, which modulates metabolic and thermogenic activity (Heldmaier *et al.* 1981). This information is subsequently used to control thermo-effector organs, like brown adipose tissue and liver, which play a role in thermogenesis. When an animal is exposed to heat stress, activation of capsaicin

cation channels (in peripheral thermoreceptors) triggers processes such as vasodilation and sweating, leading to a decrease in body temperature (Palkar *et al.* 2015). On the other hand, during cold stress, animals respond by activating multiple cation channels (including *Trpa1*, *Trpm8* and *Ucp1*) resulting in skin vasoconstriction and thermogenesis in brown adipose tissue and muscles (Palkar *et al.* 2015; Romanovsky 2018). Nevertheless, understanding of the molecular mechanisms underlying thermoregulatory processes among different species, such as strictly homeothermic and those that seasonally or daily downregulate their physiology, remain fragmentary.

While birds and mammals generally maintain a high and stable body temperature of around 40 and 36°C, respectively (Ruf and Geiser, 2015; Nowack *et al.* 2020), many reflect temporal downregulation of thermoregulatory processes (Nakamura, 2011; Boyles *et al.*, 2013). Many may enter hibernation or daily heterothermy, or any state along the continuum between these extremes and strict homeothermy (Knight *et al.* 2000; Hetem *et al.* 2016; Dammhahn *et al.* 2017; Lovegrove, 2017). Hibernation is an energy-saving physiological strategy characterized by seasonal and sometimes extreme reductions in body temperature and metabolic rate (Gautier *et al.* 2018). In some hibernators, such as in arctic ground squirrel (*Urocitellus parryii*) body temperature can drop significantly, but typically moderate temperatures of hibernacula are maintained (Knight *et al.* 2000; Hetem *et al.* 2016; Dammhahn *et al.* 2017; Lovegrove, 2017). A prevalent characteristic among hibernating mammals is upregulation of a mitochondrial enzyme, pyruvate dehydrogenase kinase 4, which inhibits the oxidation of pyruvate (conversion of pyruvate to acetyl-CoA) after glycolysis (Andrews *et al.* 1998; Buck *et al.* 2002; Andrews 2007). This interruption of glycolytic intermediates entering the Krebs cycle results in a reduction in metabolic rate, conservation of carbohydrates, and ensures that lipids serve as the primary source of ATP synthesis (Andrews 2007). A recent study on hibernating European hamsters revealed alterations in expression of 18 genes, with the most significant changes occurring in brown adipose tissue (responsible for heat generation) and the hypothalamus (responsible for thermal homeostasis; Gautier *et al.* 2018; Zhao *et al.* 2017).

At the other end of thermoregulation continuum from hibernation, in daily heterotherms, temperature and metabolism fluctuate significantly between active and inactive phases of daily cycles, typically lasting less than 24 hours (Bligh and Johnson 1973; IUPS, 2001; Geiser and Turbill C. 2009; Bastos *et al.* 2021). In the round-eared elephant shrew, normal body temperature (around 37.5°C) downregulates to as low as 9.4°C (McKechnie and Mzilikazi 2011). During daily heterothermy and arousal from it numerous tissues exhibit altered expression of deiodinases, enzymes involved in (de)activation of thyroid hormones, potentially leading to reduced availability of triiodothyronine in tissues (a thyroid hormone affecting almost every physiological process, including metabolic rate; Gautier *et al.* 2018). At the onset of the arousal, all tissues exhibited increased expression of the core clock genes (*Per1* and *Per2*), suggesting a resetting of body clock at the beginning of the active period (Gautier *et al.* 2018). In primates and rodents heat shock proteins (such as HSP60 in brown adipose tissue and HSP70 in liver) are activated to provide cytoprotection (Wu *et al.* 2015). Among the 20 most upregulated genes during early stages of daily heterothermy in Djungarian hamsters were eight genes encoding

structural proteins (including five collagen genes, *Dnha2* and *Myo15a*), and the pro-coagulation factor *Vwf* (Cubuk *et al.* 2017), aiding our understanding of the molecular underpinnings of heterothermy. Despite advances in molecular biology and the application of new tools in thermal physiology (Palkar *et al.* 2015; Faherty *et al.* 2018; Gautier *et al.* 2018; Romanovsky 2018; Andrews 2019; Chayama *et al.* 2019; Chen *et al.* 2019; Tsukamoto *et al.* 2019) significant gaps remain in understanding molecular variation behind thermoregulation in mammals. Crucially, in the context of ongoing and rapid environmental and climatic changes, it remains unclear whether more flexible thermoregulation is supported by also flexible molecular mechanisms (Carey *et al.*, 2003). If so, key thermoregulatory genes might show signatures of balancing selection maintaining adaptive variability, while core genes essential for survival could remain under purifying selection. Structurally, proteins involved in metabolic regulation may exhibit greater stability across temperature ranges, potentially through cold-adapted enzyme flexibility or chaperone-assisted folding. At the network level, flexibility could be manifested by higher connectivity among regulatory genes, enabling rapid shifts in expression, or increased modularity, allowing independent regulation of torpor and hibernation. In contrast, strict homeothermic thermoregulation may rely on more rigid mechanisms that maintain body temperature within narrow ranges despite environmental fluctuations. Although simplistic, the classification of animals to discrete traditional thermoregulation strategies (Dausmann *et al.*, 2012; Boyles *et al.*, 2013) allows comparing species that adopted extremes along the continuum of physiological thermoregulation (Lovegrove *et al.*, 2001; Glanville *et al.*, 2012). We hypothesize that at ends of the extremity, daily heterotherms and hibernators may have acquired molecular flexibility in thermoregulation-related genes that support responses to rapid environmental changes. To test these hypotheses, we compiled a list of orthologues for 54 genes known to be implicated in thermoregulation, and employed genomic data from twenty-one rodent species, to examine evolutionary patterns across the genes and genomes.

Hypothesis: Species with greater flexibility in body temperature regulation (daily heterotherms and hibernators) have evolved enhanced molecular mechanisms in thermoregulation-related genes, enabling them to better respond to rapid environmental changes.

Research questions:

- Q1 - Are the same thermoregulation-related genes present and shared across endothermic species with different thermoregulatory strategies (e.g., homeotherms, daily heterotherms, hibernators)?
- Q2 - Is the strength of positive selection on thermoregulation-related genes greater in species exhibiting greater body temperature flexibility?
- Q3 - Do positively selected amino acid sites in thermoregulation-related genes lead to conformational (structural) changes in encoded proteins?
- Q4 - Do these conformational changes more strongly affect protein thermal stability in species with flexible thermoregulation?

- Q5 - Are positively selected thermoregulation-related genes more centrally integrated in protein–protein interaction (PPI) networks in flexible species?

4.2. Materials and methods

Species and genes data

To select species for our analysis we included 21 rodent (Rodentia, all) species available in Ensembl 104 database (<https://www.ensembl.org/index.html>). For those species thermoregulation strategies were assessed based on literature (Nevo and Shkolnik 1974; Eissa *et al.* 1975; Wunder *et al.* 1977; Hudson and Scott, 1979; Tannenbaum and Pivorun, 1988; Garton *et al.* 1994; Urison and Buffenstein 1994; Knight *et al.* 2000; Shimada *et al.* 2007; Poteaux *et al.* 2008; Łapiński *et al.* 2010; McMullen and Hallenbeck, 2010; Vivanco *et al.* 2010; Ushakova *et al.* 2012; Dausmann *et al.* 2013; ZaiXue *et al.* 2013; Houtmeyers *et al.* 2016; Heldmaier *et al.* 2019; Jameson 2020; Levy *et al.* 2020). Thermoregulation strategies were classified following classic established definitions (IUPS, 2001). Species were assigned here to one of three categories based on descriptions in the literature: (1) Homeotherms: species described as homeothermic, endothermic, or mammalian without mention of heterothermy, torpor, or metabolic downregulation. (2) Daily heterotherms: species explicitly described as exhibiting 'daily torpor,' 'heterothermy,' or 'temporal down-regulation of metabolism.' Short daily bouts of torpor were always considered daily heterothermy. (3) Hibernators: species reported to undergo seasonal torpor or prolonged, multi-day torpor bouts occurring in a circannual or environmentally cued pattern, were classified as hibernation. Torpor episodes exceeding 24 hours were only classified as hibernation when occurring predictably during a defined hibernation season. When multiple terms were used in different studies, priority was given to explicit classifications, with prolonged torpor (>24h) defaulting to hibernation and short bouts (<24h) to daily heterothermy. This classification resulted in 21 species: 4 hibernators, 5 daily heterotherms, and 12 homeotherms. To establish candidate gene list, a literature review identified 54 genes related to thermoregulation, torpor, daily heterothermy and hibernation in rodents and humans (Cubuk *et al.* 2017; Gautier *et al.* 2018; Nakamura, 2011; Nakamura and Morrison, 2008; Palkar *et al.* 2015; Sieck, 2002; Wu *et al.* 2015), which were converted into Ensembl IDs, resulting in 54 mouse candidate genes (Table S1).

Genes orthologue extraction

Orthologue search was conducted using the adapted pipeline from Jovanovic *et al.* 2021, incorporating phylogenetic information where possible, from a list of 54 *Mus musculus* genes (IDs: Table S1). The R package biomaRt was implemented (Durinck *et al.* 2005) to extract orthologous coding sequences from 21 rodent species in Ensembl (release 104; Table S14). For each gene a temporary dataset was created, which contained the *Mus musculus* sequence with the `tsl1` flag from Ensembl (all splice junctions of the transcript that are supported by at least one non-suspect mRNA) and all the isoforms from the other species. Sequences were clustered based on evolutionary relationships, using the MMSeq2 (70% identity), with cluster containing *M. musculus* sequence

selected for additional analysis (Steinegger and Söding 2017). The selected sequences were stored in a DNASTringSet and, converted by R package biostrings (translate function) into an AAStringSet, containing the corresponding amino acid sequences, posteriorly saved as FASTA files. A multiple sequence alignment was created with MAFFT, from the previous AA FASTA file (Katoh *et al.* 2002) and PAL2NAL program was then used to create the codon alignment (Suyama *et al.* 2006).

Positively selected amino acid sites

To predict positively selected amino acid sites, we used a phylogenetic comparative approach, and we performed maximum likelihood analyses using codon substitution rates in CodeML (package PAML; Yang 2007). To detect positively selected sites along particular lineages the non-synonymous to synonymous substitution rate ratio (ω) was calculated for the branch-site model (Yang and Bielawski 2000; Yang 2007). It allows ω to vary across codon sites as well as among branches in a phylogenetic tree. For each gene two different models (null and alternative model) with different parameter settings are calculated and compared by the likelihood ratio test (LRT). Multiple testing was considered by the Benjamini-Hochberg method. When the LRT indicated a positive selection the Bayes-Empiric Bayes method (implemented in PAML v4.9) was used to identify codons representing a positive selection site (PSS) in the foreground branch by calculating posterior probabilities (Yang, 2007). The alignment of non-homologous positions is known to frequently cause an increase in appearance of false positives (Fletcher and Yang, 2010). Because of that, all the alignments that reported signs of positive selection were manually checked. PSS with Pvalue >0.05, and sequences that had no valid PSS after manual confirmation of the MSA, were posteriorly removed from further analyses (Table S7).

Protein structure prediction

Positively selected amino acid sites were visualized in 3D following protein structure prediction, as structures for our 34 genes were unavailable in crystallization or model predictions. Structures were predicted using Iterative Threading ASSEMBly Refinement (I-TASSER, <https://zhanglab.ccmb.med.umich.edu/I-TASSER/>), which identifies structural templates from the PDB and selects the model with the best C-score for each protein [(-5,2); Zhang 2008; Roy *et al.* 2010; Yang *et al.* 2015]. Predictions with C-scores outside this range (842) and those exceeding 1500 amino acids (95) were excluded ($p > 0.05$; Table S7). Positively selected sites were mapped on predicted structures using PyMol (<https://pymol.org/2/>). For protein-ligand docking, Ucp1 and Sirt1 structures were predicted with AlphaFold (Jumper *et al.* 2021), as they showed higher similarity to available R-ray Human structures (Zhao *et al.* 2013; Kang and Chen 2023).

Protein functional conservation

To assess whether positively selected amino acids (PSS) were located in conserved or non-conserved functional regions of our protein structures, we used the Consurf web tool (Glaser *et al.* 2003; Landau *et al.* 2005; Ashkenazy *et al.* 2010; Celniker *et al.* 2013; Ashkenazy *et al.* 2016; https://consurf.tau.ac.il/consurf_index.php). Consurf analyses

evolutionary dynamics of amino acid substitutions among homologous sequences, estimates evolutionary rates, and maps these on the structure of the protein. PSS in conserved areas suggest functionally relevant changes. The methodology was applied to all 87 predicted structures by submitting their respective MSA (from the “Positively selected amino acid sites” step) and phylogenetic tree. Analysis was done using the Bayesian method, with default settings. We considered PSS in highly conserved regions as those with a Consurf score ≥ 8 , dark pink/magenta colours, and MSA conservation $\geq 70\%$, and non-conserved sites as those with a Consurf score ≤ 2 and yellow or blue colours (Figure S3, supplementary material).

Protein-ligand docking, binding energy and melting temperature predictions

The ligands ATP (CID: 5957) and NAD (CID: 5892) were downloaded as canonical SMILES from Pubchem (<https://pubchem.ncbi.nlm.nih.gov/>, accessed July 4th, 2023), and their 3D structures were generated with hydrogens added and converted to .pdb files using Open Babel (O’Boyle *et al.*, 2011). Pymol (v2.0, Schrödinger, LLC., accessed July 4, 2023) was used to renumber atoms and remove non-HETATM entries. For Ucp1 and Sirt1, with positive selection from AlphaFold predictions, PSS were manually mutated in Pymol to consensus MSA amino acids (Table S17). Ucp1/Sirt1 sequences from *M. spretus* and *C. lanigera*, species showing no positive selection, were used as branch comparisons for species with selection signs. Human X-ray structures with ATP and NAD dockings to Ucp1 (8HBV) and Sirt1 (4I5I), respectively, identified active sites for docking predictions. Protein-ligand interactions were analysed using PLIP (Adasme *et al.*, 2021), with the best docking models selected based on HADDOCK scores (van Zundert *et al.* 2016) and binding affinity (ΔG , kcal/mol) predicted by PRODIGY-LIG (Vangone *et al.*, 2019). The influence of PSS mutations on protein stability was calculated using $\Delta\Delta G$ values ($\Delta\Delta G_{mut-wt} = \Delta G_{mut} - \Delta G_{wt}$). Positive $\Delta\Delta G$ values >0.5 indicate stabilizing variants, while negative values <-0.5 indicate destabilizing variants (Pancotti *et al.*, 2022; Table S10). Visualization of protein-ligand complexes was performed with Pymol and BIOVIA Discovery Studio Visualizer (Systèmes D., 2021), and structural homology confirmed by aligning the complexes with *H. sapiens* X-rays. Melting temperatures for all protein sequences and species were calculated at 27°C and 37°C cellular temperatures using the DeepSTABp web tool (Jung *et al.*, 2023; Table S9, Figs S5-7).

Protein-protein interaction network

Protein-protein interaction networks were constructed using all the databases integrated in the EvoPPI web tool (<http://evoppi.sing-group.org/dashboard>) by querying our candidate gene list (Table S15) in *Mus musculus*. However, due to the extensive interactor data available for *Homo sapiens* (which includes 32,700 more interactors than *M. musculus*), we opted to use *H. sapiens* as the reference species, considering the wealth of published data, as well as the homology and similarity between the studied proteins. EvoPPI is a tool that can gather and compare Protein-Protein Interaction (PPI) data from several publicly available databases for several model species (Vásquez *et al.* 2019). To do these analyses, the “query” option and the “same species” parameter were chosen, and the “*Homo sapiens*” species was selected. Then, in the “gene” field and all our 34 positively selected gene names were submitted (Table S14 and S18) resulting in

detection of 34 interactomes (Table S12 and S13). All the available interactome databases were selected (gene IDs information; Tables S13, S15-16). First and second interactors data was obtained in the same way as for our 34 PSS genes. For network visualization, Cytoscape software version 3.9.1 was used (Shannon *et al.* 2003). Clusters were created with “clusterMaker Cluster Network” application with “Community cluster, GLaY” (Su *et al.* 2010). The ClusterViz app was used to perform the score and modularity analysis within each module of the previously GLaY clustered network (Wang *et al.* 2015). Here, we used the Fast AGglomerate algorithm to identify functional modules, being this method based on the Edge Clustering coefficients (FAG-EC). The analysis was made with all the selected default values, where modularity varies between 0 (not modular) until ∞ (extremely modular), here values bigger than “2” were modular (Wang *et al.* 2015).

4.3. Results

Genes involved in thermoregulation are shared among rodents

Based on a thorough literature review we assembled a list of 54 genes (Figure 11, Attachment 1, Attachment 2).

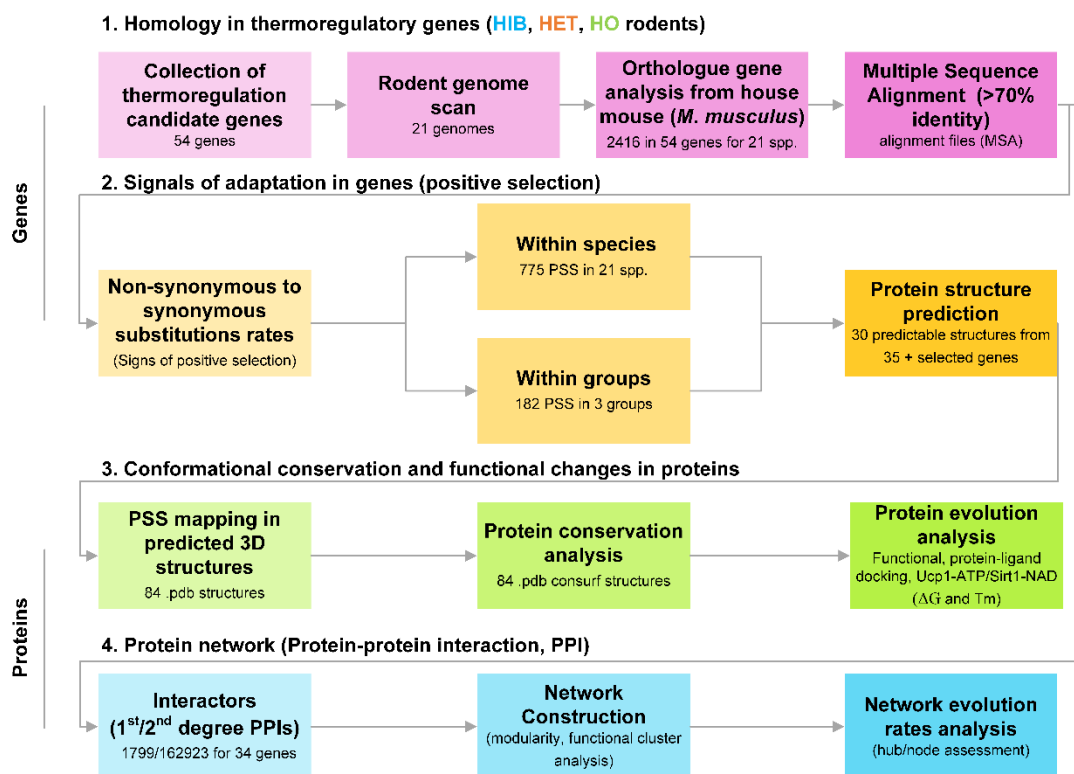


Figure 11 - Flowchart summarizing methodological processes and analyses conducted in this work on thermoregulatory strategies in 21 rodent species. Bold text represents the main methodology steps, while regular text denotes corresponding results. Colour gradients guide the reader through the accumulation of tasks, with lighter to darker tones. Green and reddish colours represent gene-related steps, while yellow and blue colours represent protein-related steps (Figure S1).

These thermoregulation-associated genes were analysed across 21 rodent species. These rodent species traditionally classified three thermoregulation strategies: homeothermy (12 species), daily heterothermy (5 species), and hibernation (4 species, Boyles *et al.*, 2013). (Figure 11, Figure 12, Attachment 1, Attachment 2).

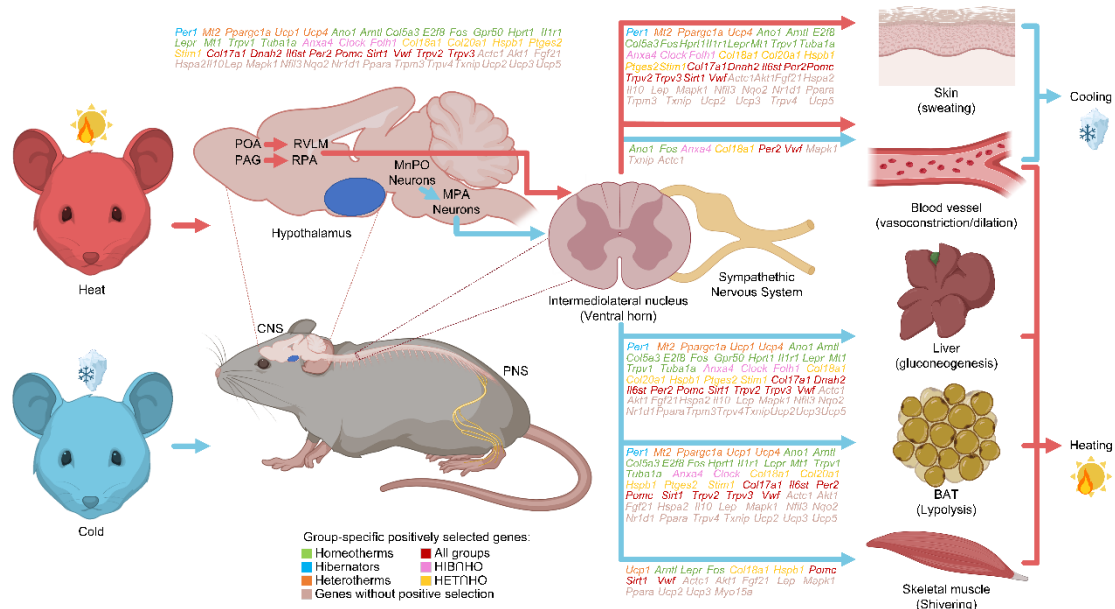


Figure 12 - Physiological pathways of thermoregulation and thermogenesis in rodents in response to heat and cold stimuli. Thermal signals are primarily processed in the hypothalamus, especially the preoptic area (POA), with heat stimuli also involving the periaqueductal grey (PAG) in the midbrain. These regions activate the rostral ventrolateral medulla (RVLM) and the raphe pallidus (RPA), which communicate via the sympathetic nervous system (through the intermediolateral nucleus of the spinal cord) to regulate vasomotor tone, heat dissipation, shivering, non-shivering thermogenesis, and brown adipose tissue (BAT) activity. Cold stimuli are processed in the anterior hypothalamus, notably in the median preoptic nucleus (MnPO), which triggers sympathetic outputs to induce thermogenesis and elevate body temperature. The figure highlights 54 studied genes, color-coded by selection signatures: green (homeotherms, HO), blue (hibernators, HIB), orange (heterotherms, HET), turquoise (HIB/HO), brown (HET/HO), magenta (all groups), and grey (no signs of selection).

Although these strategies are understood to lie along a continuum (Boyles *et al.*, 2013), we adopt the traditional classification framework to enable clearer comparisons between species with varying thermoregulatory traits. Most of those genes exhibit broad housekeeping functions, including cell cycle regulation, and are not exclusively involved in thermoregulation (Attachment 1; Joshi *et al.*, 2022), suggesting a potential link between general cellular functions and thermoregulatory adaptations. To investigate evolutionary patterns for those 54 genes, we focused on 21 rodent species available in the Ensembl database. Mapping the species by their thermoregulatory strategy in a phylogenetic tree suggests that each strategy evolved multiple times independently (Figure 13, Figure 14, Figure 15).

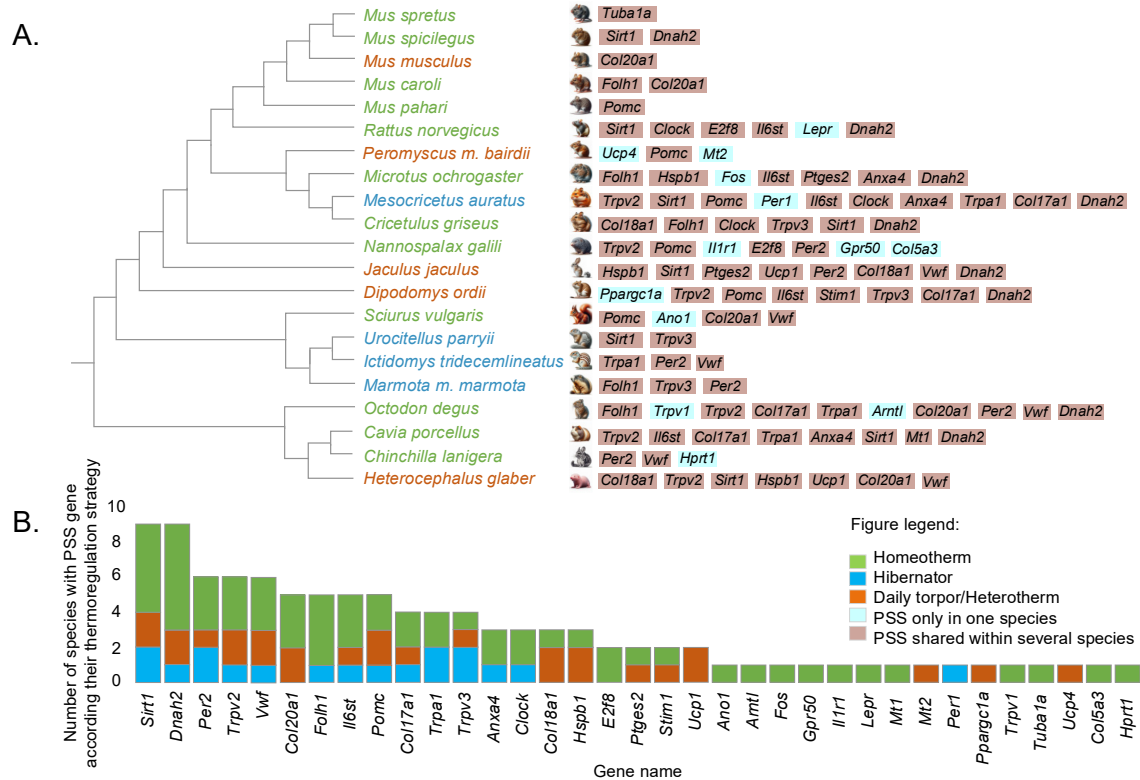


Figure 13 - Species cladogram and positive selection results. A) Cladogram of species included in analysis and their thermoregulation strategies: homeotherms (green), hibernators (cyan) and daily heterotherms (orange). Rectangles adjacent to the cladogram highlight positively selected amino acid sites (PSS): unique sites (light blue) and shared sites (pale-rose). B) Genes with significant signs of positive selection across species with different thermoregulation strategies: homeotherms (green), hibernators (blue) and daily heterotherms (orange), with gene names on the X-axis and the number of species with a specific gene selected on the Y-axis.

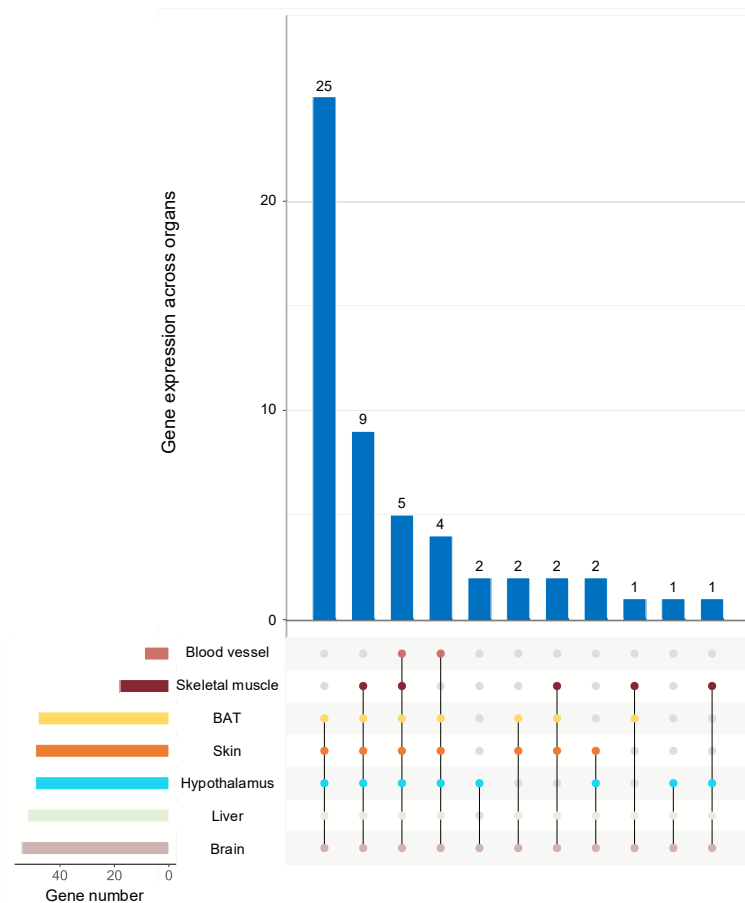


Figure 14 - Number of genes expressed across thermoregulation-related organs in *Mus musculus*. Expression and interceptions of our 54 thermoregulation-associated genes across several organs, namely in blood vessel, skeletal muscle, brown adipose tissue (BAT), skin, hypothalamus, liver and brain. Expression data depicted from Mouse gene expression database (MGI, <https://www.informatics.jax.org/expression.shtml>).

Within the genomes of 21 rodent species, we identified 2416 orthologs for our investigated genes (Figure 15, Attachment 2, see Methods).

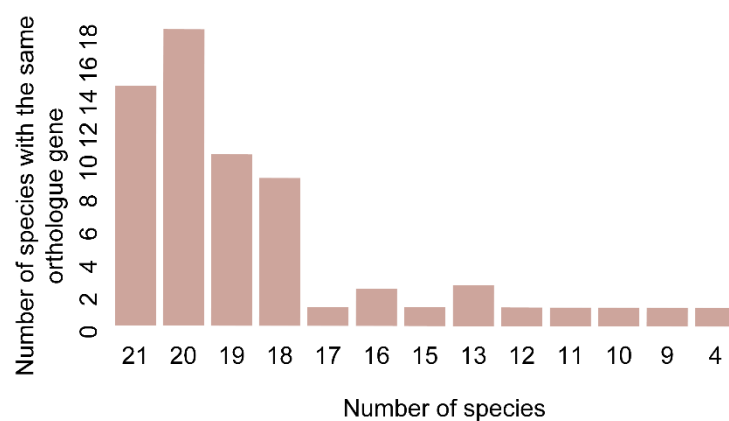


Figure 15 - Orthologue presence across species, with the X-axis representing the number of species and the Y-axis indicating the number of species sharing the same orthologue gene.

Most genes are present in all the 21 studied species, with only a few missing in one of the species (*Fgf21*, *Trpa1*, *Ppargc1a*, *Ucp5*, *Ucp3*, *Ucp2*, *Trpv3*, *E2f8*, *Trpm3*, *Trpm8*, *Arntl*, *Mapk1*, and *Il10*; Figure 15; Boyles *et al.*, 2013). To add missing orthologs we manually checked the NCBI database for 11 genes (*Col20a1*, *Sirt1*, *Il6st*, *Mt1*, *Per2*, *Gpr50*, *Nfil3*, *Lepr*, *Lep*, *Hspa2*, and *Tuba1a*), adding orthologs from a combination of Ensembl and NCBI data to complete the ortholog list to 54. Some orthologs of our candidate genes may be missing due to genome quality issues, with no single genome standing out as having significantly more missing orthologs. The overwhelming majority of genes are present in all the 21 species and 44 out of 54 genes (~81.5%) were present in at least 18 species (Figure 15).

One exception is *Gpr50* which seems to be specific to the Muroidea clade (Gupta and Suggett, 2022), including nine studied species (*Mus spretus*, *M. spicilegus*, *M. musculus*, *M. caroli*, *Rattus norvegicus*, *Peromyscus maniculatus bairdii*, *Microtus ochrogaster*, *Cricetulus griseus*, *Nannospalax galili* and *Mesocricetus auratus*). *Gpr50* is derived from melatonin receptor *Mel1c* present in amphibians and birds (Dufourny *et al.* 2008; Gautier *et al.* 2018). Mice carrying a mutation in *Gpr50* displayed an enhanced propensity to fasting-induced physiological down-regulation (Bechtold *et al.* 2012; Ivanova *et al.* 2008). While its expression varies during arousal from daily heterothermy (torpor), its expression remains stable across hibernation stages in European hamsters (*Cricetus cricetus*; Gautier *et al.*, 2018). In homeotherms this gene is mostly associated with heterodimerization and inhibition of melatonin receptor genes MT1 and MT2 (Levoye *et al.* 2006). The second exception is *Myo15a*, which is restricted to the *Mus* genus, where one of the analysed species, *M. musculus*, is confirmed to express daily heterothermy (Hudson and Scott, 1979; Lo Martire *et al.*, 2020; Hare *et al.*, 2024). The gene was detected in four out of five *Mus* species (although it exhibited low sequence similarity in *M. spicilegus*: <70%; Figure 3A; Cubuk *et al.*, 2017). This gene was linked to daily temperature downregulation also in Djungarian hamsters (Cubuk *et al.*, 2017). While the genome of the Djungarian hamster is not available in Ensembl, and hence was not included in analysis, we did not detect *Myo15a* in the two other hamsters in our study, the Chinese and golden hamsters, likely due to high sequence divergence hindering ortholog detection. Presence and conservation of *Myo15a* in *M. spretus*, *M. caroli*, and *M. pahari* suggests its potential role in torpor expression in *Mus* (Figure 13). Indeed, several laboratory strains of *M. musculus* showed daily body temperature downregulation (ranging from the normothermic 39.3 ± 0.3 °C to torpid 16–19°C; Hudson and Scott, 1979), but it remains unknown how widespread torpor is in other *Mus* species.

A total of 54, 52 and 54 genes were found in daily heterotherms, hibernators and homeotherms. Interestingly, *Il1r1* and *Myo15a* genes were not detected in any of our four hibernator species, suggesting they may have adapted to function without them. In *Il1r1* knockout mice, the absence of IL-1 β signalling prevents reduction in hippocampal cell proliferation caused by sleep deprivation (Mueller *et al.* 2014). This lack of *Il1r1* orthologs in hibernators could be an adaptation to prolonged torpor, helping to avoid IL-1 β 's inflammatory and neurogenesis-suppressing effects, thereby protecting their brains and conserving energy during extended “sleep” cycles. Similarly, *Myo15a* knockout mice experience congenital deafness and balance issues due to disrupted stereocilia

formation in hair cells (Kanzaki *et al.* 2006). The absence of *Myo15a* orthologs in hibernators might indicate alternative mechanisms supporting essential hearing and balance, aligning with the sensory demands of their torpor and arousal cycles. This adaptation could reflect an evolutionary shift in how hibernators manage sensory functions through seasonal cycles.

Overall, most of the 54 genes studied are shared among species with different thermoregulation strategies, supporting previous suggestions that the mechanisms of thermoregulation are ancient (Grigg *et al.* 2004). None of the genes were exclusive to species of one of the three thermoregulatory groups. Even the phylogenetically restricted genes, *Gpr50* and *Myo15a*, are present in genus of at least two thermoregulatory types. This suggests that the strategies employed by the species for regulating their body temperature can change relatively rapidly without evolving new genes. Evolutionary changes in particular genes could potentially allow species to switch between thermoregulatory strategies within relatively short evolutionary time, making thermoregulation a fast-evolving trait.

Stronger signs of positive selection among daily heterotherms

We investigated signs of adaptive evolution by calculating ratios of non-synonymous to synonymous substitutions (Yang, 1997) for the 54 genes across all 21 rodents, using a branch-site model (Yang and Bielawski 2000). We detected signs of positive selection (ratios of non-synonymous to synonymous substitutions; $\omega = dN/dS > 1$) in 35 out of 54 genes, in at least one species (Table 8, Attachment 3, Attachment 4, Attachment 5).

Table 8- Estimated signals of selection in thermoregulatory genes. Summary of forebranch analysis results across (PSS), group-specific genes, genes shared among thermoregulation strategies and genes shared between hibernators and homeotherms (HIB $\cap$ HO) or daily heterotherms and homeotherms (HET $\cap$ HO). Parenthesized values represent species count or percentage of positively selected genes (tables S4 and S5).

	No. genes with PSS	total PSS	PSS/n° genes with PSS	Normalized PSS number	Specific genes within thermoregulation strategies	shared genes among all strategies	HIB $\cap$ HO	HET $\cap$ HO	HIB $\cap$ HET
Hibernators (4)	14 (40%)	125	8.93	31.25	1 (2.9%)				
Daily heterotherms (5)	18 (51.4%)	203	11.27	40.60	4 (11.4%)	9 (25.7%)	4 (11.4%)	5 (14.3%)	0 (0%)
Homeotherms (12)	30 (85.7%)	445	14.83	37.91	12 (34.3%)				

Each of the 21 species studied showed signs of positive selection in at least one gene (Figure 13). While 13 positively selected genes were unique to a single but variable species (Figure 13, cyan), most genes (22) showed positive selection in species from at least two thermoregulation groups (Figure 13). There were eight genes that showed signs of selection in species representing all three strategies, such as *Sirt1*, suggesting no clear group-specific gene selection. The only potential exception is *Ucp1*, which was found to be under positive selection in heterotherms but not in the other two groups. However, we cannot interpret this as a group specific signal as *Ucp1* was under selection only in two out of 5 heterothermic species (Attachment 3).

We tested if any of the groups experienced more adaptive changes in thermoregulatory genes than the others, hypothesizing that natural selection on thermoregulation might be stronger in species with more extreme temperature changes. Positive selection signals were detected in 30 genes in homeotherms, 18 in daily heterotherms and 14 in hibernators, which corresponds to ~3.5 genes per heterotherm and hibernator, but only ~2.5 genes per homeothermic species. Calculating the average number of positively selected sites (PSS) per positively selected gene and normalizing for the number of species within the thermoregulatory group, we found that daily heterotherms held the highest number of PSS (averaged: 40.60), followed by homeotherms (37.91) and hibernators (31.25). Taken together, these results suggest that slightly more genes and amino acids per gene show signs of selection in daily heterotherms (Table 8). Though this pattern alone does not conclusively demonstrate greater molecular flexibility. Further analysis is needed to disentangle the relative influence of phylogenetic relationships and ecological factors on the pattern. While daily heterotherms often experience environmental fluctuations, it remains unclear whether these fluctuations directly drive stronger selection on thermoregulatory genes or if other selective pressures play a more dominant role.

Overall, a relatively high proportion of genes with signs of positive selection among the 54 thermoregulatory genes was found. While previous studies estimated that about 1-3% of genes are under positive selection in rodents, we detected at least one positively selected gene in each species, ~1.8% (1 in 54 genes), with most species having more than one gene under positive selection. Such high proportion might reflect that rodents are more exposed to selective pressures from thermally fluctuating environmental conditions (e.g. the usually small size of rodent results in their limited mobility, high rate of surface heat losses and fast life histories and physiologies). Positive selection of genes involved in thermoregulation, as a signal of adaptation to cold environments, have also been observed in other mammals, such as bats (Zu *et al.* 2018). In summary, we could not reveal genes that were clearly only positively selected in one of the thermoregulatory groups. Moreover, in genes with signs of positive selection in more than one species, the selected sites differed between the species (Figure 15, Table 8). Yet, slightly more adaptive changes were found in genes in heterothermic species. Therefore, it is possible that along thermoregulation continuum heterotherms may undergo stronger adaptive evolution due to their need to cope with a greater range or changes in environmental conditions.

Functional changes of thermoregulatory proteins in daily heterotherms

Lowering body temperature during daily heterothermy or hibernation may affect protein folding and structural stability. We investigated the impact of positively selected amino acid changes on protein conformation. We predicted and analysed protein structures for 30 genes showing positive selection across the 21 species (see Methods). We evaluated 439 positively selected sites in proteins shorter than 1500 amino acids for conserved structure (value >8 in Consurf and >70% sequence conservation, supplementary material). Most changes affected non-conserved regions (417 sites in 20 genes, Attachment 6). Only 22 positively selected sites were found in conserved protein regions of 10 thermoregulatory genes, changing proteins in multiple different species of the three thermoregulatory groups (Table 9, Attachment 7).

Table 9 -Estimated signals of selection in conserved sites of amino acid. Summary of conserved amino acid sites under positive selection across species with different thermoregulation strategies and their positions on protein structures. Abbreviations: Term. (thermoregulation type; HET = daily heterotherm, HIB = hibernator, HO = homeotherm). Includes gene name, amino acids with positive selection on moderately to highly conserved sites (CPSS) and type of these sites in rodents' proteins

Species	Term.	gene	amino acids	CPSS	type
<i>Heterocephalus glaber</i>	HET	<i>Col18a1</i>	PHE ¹⁰²⁵ , SER ¹⁰²⁸	2	α-helix
<i>Sciurus vulgaris</i>	HO	<i>Col20a1</i>	TRP ⁶⁷¹	1	β-strand
<i>Mesocricetus auratus</i>	HIB	<i>Trpv2</i>	ASN ⁶⁴⁸	1	α-helix
<i>Crices griseus</i>	HO	<i>Sirt1</i>	SER ²⁶⁵	1	α-helix
<i>Cavia porcellus</i>	HO	<i>Sirt1</i>	PHE ⁵⁷⁵	1	loop
<i>Rattus norvegicus</i>	HO	<i>Sirt1</i>	PRO ⁵⁵⁵	1	loop
<i>Dipodomys ordii</i>	HET	<i>Pomc</i>	PRO ⁹	1	loop
<i>Rattus norvegicus</i>	HO	<i>Il6st</i>	MET ¹⁹⁴ , HIS ⁶⁰²	2	loop, β-strand
<i>Sciurus vulgaris</i>	HO	<i>Ano1</i>	ARG ⁸⁹⁴	1	loop on the protein's "arm"
<i>Cavia porcellus</i>	HO	<i>Trpa1</i>	MET ²⁹⁰	1	α-helix
<i>Chinchilla lanigera</i>	HO	<i>Per2</i>	VAL ¹⁸⁶ , ALA ⁴⁶¹ , ARG ⁸⁶³	3	loops
<i>Octodon degus</i>	HO	<i>Per2</i>	ILE ⁷⁸⁸	1	loop
<i>Ictidomys tridecemlineatus</i>	HIB	<i>Per2</i>	GLN ²⁷⁷ , ALA ²⁸⁰ , ASN ⁵⁰⁹ , ALA ⁵¹⁵	4	loops
<i>Mus spretus</i>	HO	<i>Tuba1a</i>	ILE ¹⁸⁶ and THR ²⁶²	2	α-helix, loop

Sirt1 and *Per2* showed PSS in conserved regions in most species, with *Per2* showing up to four potentially functional changes (Table 9, Figure 16, Attachment 6).

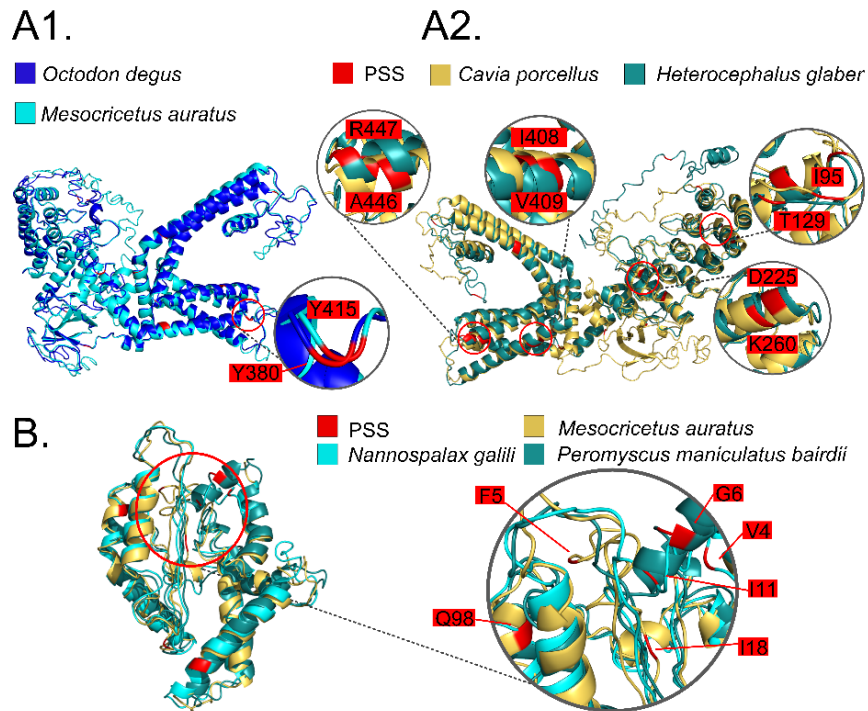


Figure 16 - A1. Trpv2 protein structures, showing overlap, in *Mesocricetus auratus* whose structure is painted in cyan and *Octodon degus* painted in dark blue A2. Trpv2 protein structures, showing overlap, in *Cavia porcellus* whose structure is painted in yellow-orange and *Heterocephalus glaber* painted in deep teal. Here, we focused on frontal and back with two visible clusters of overlapping PSS (painted in red) present in α -helices are shown in detail. B. Pomc protein cluster overlap, PSS in *Mesocricetus auratus* (4 PSS) whose structure is painted in yellow-orange, *Nannospalax galili* (3 PSS) painted in cyan and *Peromyscus maniculatus bairdii* (1 PSS) painted in deep teal. Upper plane of the three structures, presents a cluster of positively selected amino acid sites (painted in red), indicative of parallel selection.

***Ucp1* and *Sirt1* activation – a case study**

We focused on *Ucp1* (Figure 17, Table 10) and *Sirt1* (Figure 18, Table 11) to explore the effects of positive selection on protein function activation and thermal stability, as *Ucp1* was exclusively positively selected in daily heterotherms, while *Sirt1* was positively selected across groups in conserved sites (Figure 13).

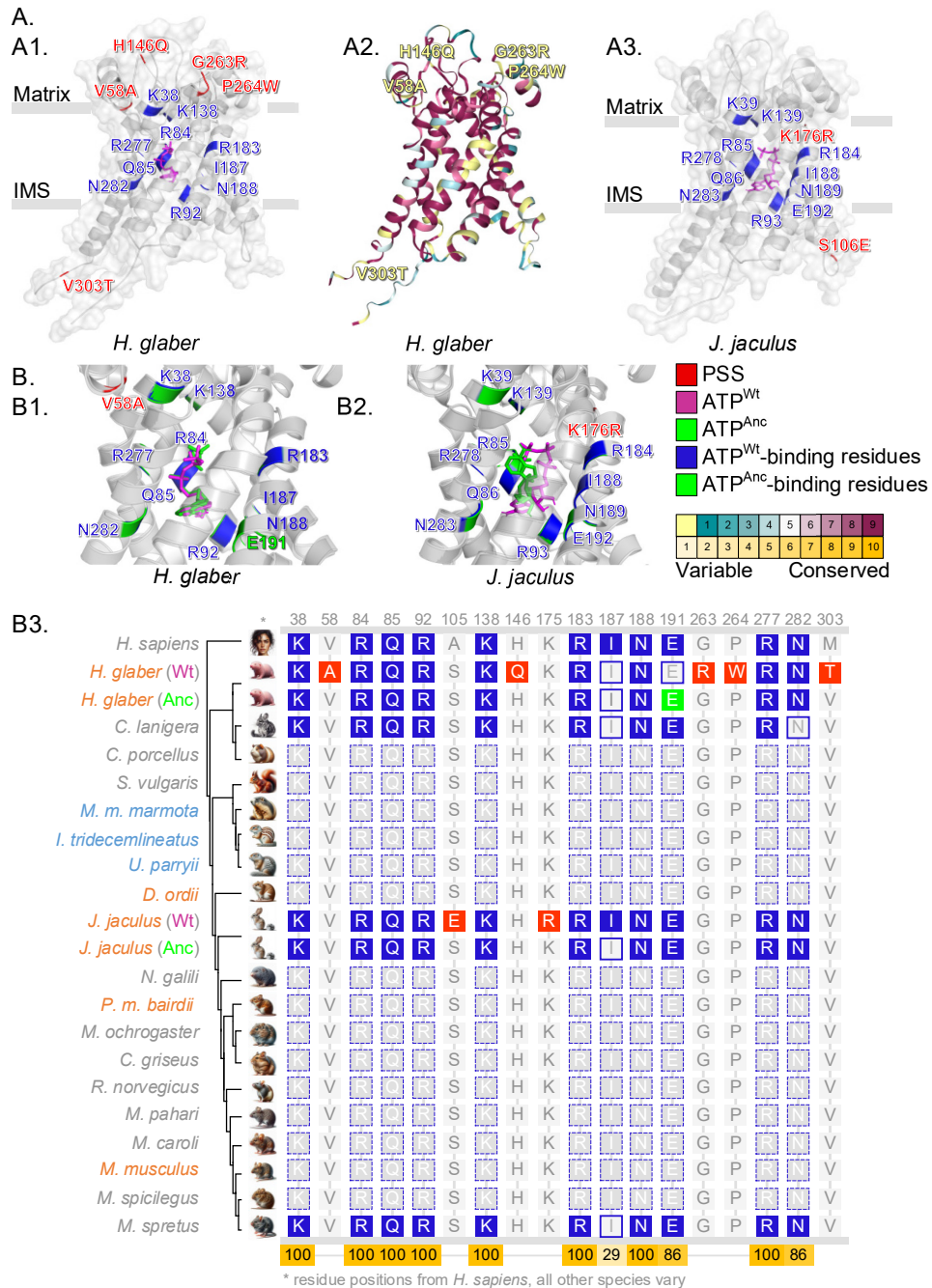


Figure 17 – Ucp1 protein activation evolution in daily heterotherm species. A) Ucp1-ATP docking in daily heterothermic species: Ucp1 structures for *Heterocephalus glaber* (A1) and *Jaculus jaculus* (A3) show ATP-binding sites in blue, derived ATP in magenta, and positively selected sites in red. Conservation analysis for *H. glaber* (A2) highlights non-conserved selected sites in yellow, with the structure's colour scale from yellow (not conserved) to deep purple (extremely conserved). B) Evolution of Ucp1-ATP binding sites: For *H. glaber* (B1) and *J. jaculus* (B2), derived (blue) and ancestral (green) ATP-binding sites are shown with ATP in magenta (derived) and green (ancestral); positively selected sites in red. Panel B3 summarizes binding site evolution, with blue squares indicating active ATP-binding, green for ancestral presence, empty for loss, red for positive selection, and yellow squares for conservation scores.

Table 10 - Protein-ligand docking results for Ucp1-ATP. C stands for complex, T for thermoregulatory strategy, Spp for species, Type for protein type, Z for Z-score, H for Haddock score, ΔG for binding energy (Kcal/mol), $\Delta\Delta G_{spp}$ for variation of binding energy of each species ($\Delta\Delta G = \Delta G_{Anc} - \Delta G_{Wt}$), $\Delta\Delta G_{group}$ for variation of binding energy of each species with positive selection compared with the closest control species (without signs of positive selection, *C. lanigera* or *M. spretus*), Tm_{27} for melting temperature at 27°C of cell temperature, Tm_{37} for melting temperature at 37°C of cell temperature, ΔTm_{37-27} for difference between Tm_{37} and Tm_{27} , $\Delta Tm_{27Anc-Wt}$ for variation of Tm at 27°C between types, $\Delta Tm_{37Anc-Wt}$ for variation of Tm at 37°C between types, % D. Tm_{27} from Tm_{37} , for the % of denaturation that corresponds to Tm_{27} on the Tm_{37} melting curve, $\Delta\%DTm_{27Anc-Wt}$ for the variation between types of the % of denaturation at Tm_{27} , % D. 27°C, for % of denaturation at 27°C and % D. 37°C, for % of denaturation at 37°C.

C	T	Spp	Type	Z	H	RMSD	ΔG	$\frac{\Delta \Delta G_{sp}}{p}$	$\frac{\Delta \Delta G_{gro}}{up}$	Tm ₂₇	Tm ₃₇	$\frac{\Delta Tm_{37} - \Delta Tm_{27}}{T_{Anc-Wt}}$	% D. Tm ₂₇ from Tm ₃₇	$\Delta\%$ D.Tm 27Anc-Wt	% D. 27°C	% D. 37°C		
Ucp1-ATP	HET	H. glaber	Wt	-1.60	-47.6 +/- 0.5	0.4 +/- 0.1	-6.05	NA	-0.26	50.08	52.24	2.16	NA	34.77	NA	0.042728	1.068294	
			Anc	-1.5	-46.4 +/- 0.4	0.4 +/- 0.3	-5.98	0.07	-0.19	50.88	52.14	1.27	-0.09	40.83	6.06	0.043959	1.09874	
		J. jaculus	Wt	-2	-56.3 +/- 2.1	0.3 +/- 0.2	-5.98	NA	0.04	50.9095	52.3436	1.43	NA	40.15	NA	0.041532	1.038701	
			Anc	-2	-51.0 +/- 2.5	0.4 +/- 0.2	-5.95	0.03	0.07	50.46	52.19	1.73	-0.15	38.10	-2.06	0.042622	1.066788	
	HO	C. lanigera	Wt - ref	-0.9	-47.1 +/- 0.6	0.4 +/- 0.3	-5.79	NA	NA	50.12	52.11	2.00	NA	36.08	NA	0.043893	1.097817	
			M. spretus	Wt - ref	-1.7	-49.9 +/- 0.4	0.4 +/- 0.2	-6.02	NA	NA	50.53	52.11	1.58	NA	38.77	NA	0.043893	1.097817
		H. sapiens	Wt - ref	(x-ray)	-2.2	-66.8 +/- 0.6	0.2 +/- 0.2	-6.45	NA	NA	52.15	52.55	0.40	NA	47.16	NA	0.037974	0.952317

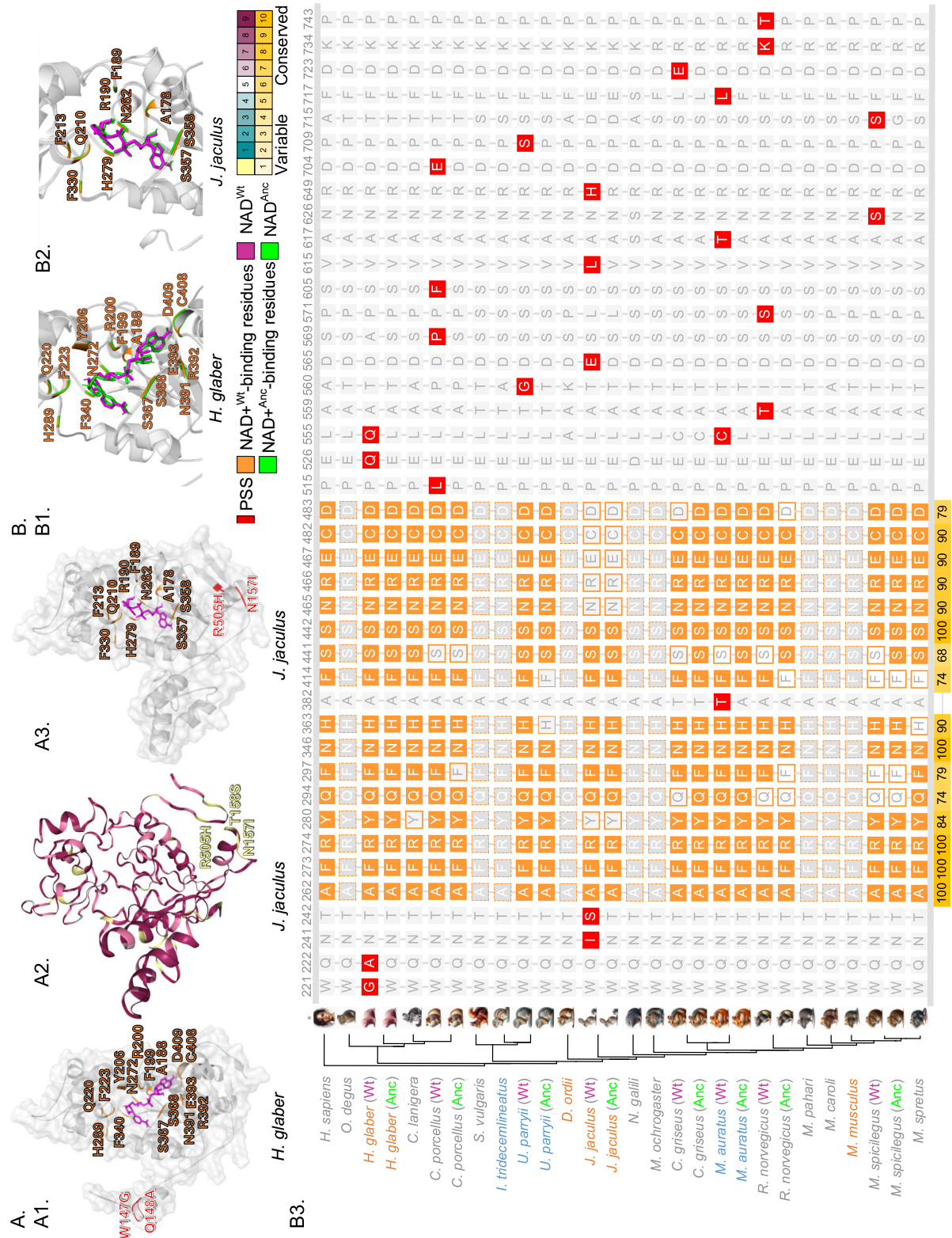


Figure 18 - Sirt1 protein activation evolution across species. A) Sirt1-NAD Docking in *H. glaber* and *J. jaculus*: Sirt1 structures for *H. glaber* (A1) and *J. jaculus* (A3) show NAD-binding sites in orange, derived NAD in magenta, and selected sites in red. Conservation for *H. glaber* (A2) highlights low conservation (yellow) in selected sites. B) Evolution of Sirt1-NAD Binding Sites: Structures for *H. glaber* (B1) and *J. jaculus* (B2) show derived (orange) vs. ancestral (green) NAD-binding sites; panel B3 summarizes NAD-binding site evolution with orange for active binding, green for ancestral presence, empty for loss, red for positive selection, and yellow squares for conservation scores.

Table 11 - Protein-ligand docking results for Sirt1-NAD. C stands for complex, T for thermoregulatory strategy, Spp for species, Type for protein type, Z for Z-score, H for Haddock score, ΔG for binding energy (Kcal/mol), $\Delta\Delta G_{\text{spp}}$ for variation of binding energy of each species ($\Delta\Delta G = \Delta G_{\text{Anc}} - \Delta G_{\text{Wt}}$), $\Delta\Delta G_{\text{group}}$ for variation of binding energy of each species with positive selection compared with the closest control species (without signs of positive selection, *C. lanigera* or *M. spretus*), T_{m27} for melting temperature at 27°C of cell temperature, T_{m37} for melting temperature at 37°C of cell temperature, ΔT_{m37-27} , for difference between T_{m37} and T_{m27} , $\Delta T_{m27\text{Anc-Wt}}$ for variation of T_m at 27°C between types, $\Delta T_{m37\text{Anc-Wt}}$ for variation of T_m at 37°C between types, % D. T_{m27} from T_{m37} , for the % of denaturation that corresponds to T_{m27} on the T_{m37} melting curve, $\Delta\%DT_{m27\text{Anc-Wt}}$ for the variation between types of the % of denaturation at T_m 27, % D. 27°C, for % of denaturation at 27°C and % D. 37°C, for % of denaturation at 37°C.

C	T	Spp	Type	Z	H	RMSD	ΔG	$\Delta\Delta G_{\text{sp}}$	$\Delta\Delta G_{\text{gro}}$	T_{m27}	T_{m37}	ΔT_{m37-27}	$\Delta T_{m37\text{Anc-Wt}}$	% D. T_{m27} from T_{m37}	$\Delta\%DT_{m27\text{Anc-Wt}}$	% D. 27°C	% D. 37°C
Sirt1-NAD	HET	<i>H. glaber</i>	Wt	-1.30	-67.8 +/- 2.6	0.6 +/- 0.4	-7.83	NA	-0.41	44.9	45.6	0.64	NA	45.56	NA	0.28854	6.81488
			Anc	-2.2	-67.7 +/- 5.2	0.5 +/- 0.3	-7.75	0.08	-0.33	45.0	45.6	0.62	0.01	45.57	0.00	0.28876	6.81699
		<i>J. jaculus</i>	Wt	-1.3	-67.2 +/- 0.6	0.5 +/- 0.2	-8.00	NA	-0.33	46.0	47.2	1.23	NA	41.27	NA	0.17471	4.24089
			Anc	-1.5	-67.9 +/- 2.5	0.3 +/- 0.2	-7.97	0.03	-0.30	46.2	47.3	1.12	0.07	41.99	0.72	0.17521	4.24651
		<i>C. porcellus</i>	Wt	-1.2	-63.0 +/- 5.7	0.6 +/- 0.5	-7.82	NA	-0.40	45.9	46.5	0.61	NA	45.59	NA	0.21456	5.15929
			Anc	-1.1	-65.3 +/- 2.7	0.6 +/- 0.3	-7.84	-0.02	-0.42	45.8	46.4	0.60	-0.08	45.59	0.00	0.22104	5.30611
		<i>U. parryii</i>	Wt	-1.5	72.7 +/- 1.3	0.5 +/- 0.3	-7.87	NA	-0.45	45.7	46.2	0.57	NA	45.58	NA	0.23451	5.61093
			Anc	-0.4	-70.2 +/- 1.3	0.6 +/- 0.2	-7.57	0.30	-0.15	45.7	46.3	0.58	0.03	45.58	0.00	0.23479	5.61378
		<i>C. griseus</i>	Wt	-1.9	-54.8 +/- 4.3	0.7 +/- 0.1	-7.97	NA	-0.30	46.0	46.9	0.90	NA	43.41	NA	0.19673	4.74713
			Anc	-1.5	-59.6 +/- 0.9	1.0 +/- 0.2	-7.83	0.14	-0.16	46.0	46.9	0.90	0.01	43.42	0.00	0.19680	4.74799
	HO	<i>R. norvegicus</i>	Wt	-1.9	-68.6 +/- 5.0	0.6 +/- 0.3	-7.87	NA	-0.20	47.0	48.1	1.04	NA	42.74	NA	0.13858	3.38968
			Anc	-1.9	-53.1 +/- 3.2	0.5 +/- 0.1	-7.91	-0.04	-0.24	46.7	47.9	1.24	-0.11	41.31	-1.44	0.14261	3.48573
		<i>M. spicilegus</i>	Wt	-0.5	-43.0 +/- 1.2	0.9 +/- 0.0	-7.99	NA	-0.32	47.4	48.3	0.89	NA	43.47	NA	0.12673	3.11177
			Anc	-0.6	-44.1 +/- 1.2	0.8 +/- 0.2	-8.10	-0.11	-0.43	47.5	48.4	0.95	0.13	42.76	-0.71	0.12326	3.02720
		<i>C. lanigera</i>	Wt - ref	-1.6	-54.8 +/- 0.8	0.6 +/- 0.3	-7.42	NA	NA	47.1	47.8	0.72	NA	44.90	NA	0.15136	3.68965
			Wt - ref	-1.8	-64.1 +/- 0.9	0.6 +/- 0.1	-7.67	NA	NA	46.2	47.0	0.79	NA	44.15	NA	0.18523	4.48542
		<i>H. sapiens</i>	Wt - ref (x-ray)	-1	-38.7 +/- 0.9	0.3 +/- 0.0	-7.35	NA	NA	45.8	46.3	0.59	NA	45.59	NA	0.22763	5.45601
			Wt	-1.3	-67.3 +/- 0.6	0.4 +/- 0.3	-7.82	NA	-0.15	47.1	47.9	0.78	NA	44.18	NA	0.14707	3.58808
		HIB <i>M. auratus</i>	Wt	-1.3	-67.3 +/- 0.6	0.4 +/- 0.3	-7.82	NA	-0.15	47.1	47.9	0.78	NA	44.18	NA	0.14707	3.58808
			Anc	-1.2	-69.4 +/- 2.1	0.5 +/- 0.3	-7.80	0.02	-0.13	47.1	47.9	0.80	-0.03	44.18	0.00	0.14689	3.58591

We hypothesize that positive selection increases protein functional and thermal stability. For functional stability, we analysed active/passive sites, PSS impact, Root mean square deviation of atomic positions (RMSD), and binding energy variation ($\Delta G/\Delta\Delta G$) for protein-ligand complexes activated by adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD). In addition, to uncover the impact of the positive selection on folding of these proteins and their functional activation we made a comparative analysis, where we tested positively selected proteins (Wild type (Wt)) for Ucp1 and Sirt1, alongside their ancestral states (Anc), obtained by reverting the amino acid sequence *in-silico* to the consensus amino acids of the Multiple Sequence Alignment (MSA) across

21 species. Phylogenetic controls included Wt sequences of orthologs without positive selection from *M. spretus* and *C. lanigera*. Docking comparisons were conducted for thermoregulation groups: Wt (selected) vs. Wt (not selected), Wt (selected) vs. Anc, and Wt (not selected) vs. Anc. Thermal stability was assessed by the quantity of denatured protein (indicating structural damage, %) and melting temperature at cellular temperature of 27°C (torpid) and at 37°C (normothermic; Ruf & Geiser, 2015; Nowack *et al.*, 2020).

Ucp1 is an essential regulator of non-shivering thermogenesis in placental mammals (Kang and Chen, 2023), that has been evolutionary conserved across various thermoregulatory groups, with evidence of its evolution before the emergence of endothermy in the common ancestor of Teleostomi (Gaudry *et al.*, 2018). ATP activation of *Ucp1*, crucial for ATP-dependent heat production, displayed some site-specific adaptations across species (Table 4). We found that the active site Ile¹⁸⁷, which is crucial for *Ucp1*'s ATP-binding function (Kang and Chen, 2023), is lost in all analysed species except *J. jaculus*, and that Glu¹⁹¹ is absent in *H. glaber* (Figure 17, Table 10), suggesting that positive selection in heterothermic species might influence ATP binding efficiency and thermogenesis capabilities. Structural alignment of *Ucp1*-ATP complexes across species revealed good fit (with similar root mean square deviation, RMSD <0.5). Tight structural alignment and *Ucp1*-ATP binding is well-conserved across species, regardless of thermoregulatory strategy. All *Ucp1*-ATP complexes exhibited negative binding energies (ΔG), indicating spontaneous ATP binding to *Ucp1* ($\bar{x}$: -5.96 kcal/mol, range: -5.79 to -6.05 kcal/mol). No differences in binding energy variation were observed between daily heterotherms and homeotherms, suggesting that the stability of the *Ucp1*-ATP complex remains robust across thermoregulatory groups (Attachment 8). *Ucp1* protein denaturation, at both 27°C and 37°C, was slightly lower in daily heterotherms than in control homeotherms (*J. jaculus* and *H. glaber* versus *M. spretus* and *C. lanigera*), suggesting marginally better thermal stability of *Ucp1*-ATP complexes in heterotherms (Table 12). *Ucp1* proteins with positively selected sites in heterothermic species showed slightly lower denaturation compared to non-selected wild types (Table 12), suggesting that positive selection could enhance the protein's thermal stability. Melting temperature simulations at 27°C and 37°C revealed that the protein model developed for 37°C for heterothermic *Ucp1*, was more stable when compared to reconstructed ancestral and control homeothermic proteins (those with no sign of selection: *Mus spretus* and *Chinchilla lanigera*). Difference in more than 1.4°C of melting temperature in simulated proteins at 27°C and 37°C indicates a significant temperature sensitive activation of *Ucp1* in daily heterotherms (Table 12). This suggests that *Ucp1* is more readily activated at higher temperatures, potentially reflecting species-specific adaptations in the ATP-dependent activation of *Ucp1*.

Flexibility of thermoregulation in mammals: Molecular evolution, gene dynamics and ecological drivers

Table 12 - Summary results of functional analyses made across docking comparisons and protein forms (Wild type (Wt), Ancestral (Anc)) for Ucp1-ATP and Sirt1-NAD complexes across thermoregulation groups (abbreviations: HET: heterotherm, HIB: hibernator, and HO: homeotherm; ΔG : binding energy; ΔT_m : variation in melting temperature between 37 and 27°C.)

protein-ligand analyses	Ucp1	Sirt1
Species	HETs with positive selection: <i>H. glaber</i> and <i>J. jaculus</i> HO controls (no selection): <i>M. spretus</i> and <i>C. lanigera</i>	HETs with positive selection: <i>H. glaber</i> and <i>J. jaculus</i> HOs with positive selection: <i>C. porcellus</i> , <i>U. parryii</i> , <i>C. griseus</i> , <i>R. norvegicus</i> , <i>M. spicilegus</i> HIBs with positive selection: <i>M. auratus</i> HO controls (no selection): <i>M. spretus</i> and <i>C. lanigera</i>
PSS impact	of the 11 active sites, Ile ¹⁸⁷ was lost in all species, except <i>J. jaculus</i> has unique PSS Arg ¹⁷⁵ . Glu ¹⁹¹ lost in <i>H. glaber</i> , potentially linked to the unique PSSs Arg ²⁶³ and/or Trp ²⁶⁴ .	Several of the 16 active sites not detected, with no clear thermoregulatory or phylogenetic pattern
Structures	Wild type (Wt): extant structure, with or without positive selection Ancestral (Anc): consensus-based model, manually mutated at positively selected sites	Wild type (Wt): extant structure, with or without evidence of positive selection Ancestral (Anc): consensus-based model, manually mutated at positively selected sites
Docking comparisons	4 HETs (2 Wt, 2 Anc) and 2 HOs (2 comparison species)	4 HETs (2 Wt, 2 Anc), 12 HOs (7 Wt, 2 comparison species, 5 Anc), and 2 HIBs (1 Wt, 1 Anc)
RMSD	0.2 to 0.4	0.3 to 1.0
Binding energy (ΔG)	ΔG ($\bar{x}$: -5.96, range: -5.79 to -6.05 kcal/mol), indicating spontaneous binding of ATP	ΔG ($\bar{x}$: -7.84, range: -7.42 to -8.10 kcal/mol), indicating spontaneous binding of NAD
Binding energy variation ($\Delta\Delta G$)	No significant differences between HETs and control HOs	No significant differences between thermoregulatory groups and control HOs
Protein denaturation (%) at 27°C and at 37°C (depicted from melting curves)	At 27°C and at 37°C HETs: ~0.043%; ~1.068% Control HOs: ~0.044; ~1.098	At 27°C and at 37°C HETs: at ~0.232%; ~5.530% HOs: ~0.176%; ~4.248% HIBs: ~0.146%; ~3.587%
Protein denaturation difference	Wt positively selected Ucp1 proteins in HETs: ~0.042% at 27°C and ~1.053% at 37°C Anc Ucp1 proteins in HETs: ~0.043% at 27°C and ~1.083% at 37°C control HO Ucp1: ~0.044% at 27°C and ~1.098% at 37°C	Wt positively selected Sirt1: ~0.190% at 27°C and 4.583% at 37°C non-selected Wt Sirt1: ~0.168% at 27°C and ~4.088% at 37°C. Anc Sirt1: ~0.191% at 27°C and ~4.604% at 37°C
T _m simulations	Positively selected in HETs: ~52.24°C at 27°C ~52.34°C at 37°C Anc in HETs: 52.14°C at 27°C and 52.19°C at 37°C control HO: 52.14°C at 27°C and ~52.11°C at 37°C	Sirt1 proteins across groups: at 27°C ($\bar{x}$: 46.3, range 44.98-47.53°C) and 37°C ($\bar{x}$: 47.2, range 45.62-48.48°C) At 37°C: Positively selected in HETs: 45.62 <i>H. glaber</i> and 47.26°C <i>J. jaculus</i> Ancestral in HETs: 45.64 °C <i>H. glaber</i> and 47.33°C <i>J. jaculus</i> Positively selected s in HOs: 46.29, 46.90 and 48.35°C; <i>U. parryii</i> / <i>C. griseus</i> / <i>M. spretus</i> Anc in HOs: 46.32, 46.91 and 48.48°C <i>U. parryii</i> / <i>C. griseus</i> / <i>M. spretus</i>) Positively selected in HIB: 47.93°C Ancestral in HIB: 47.90°C
ΔT_m (37°C - 27°C)	$\Delta T_m > 1.4^\circ\text{C}$ (statistically significant) in all species, suggesting greater Ucp1 stability at 37°C More variation observed in HETs, range: 1.27–2.16°C than in control HOs: 1.58–2.00°C	$\Delta T_m < 1^\circ\text{C}$ in most rodents, suggesting non-significant thermal shift, except in <i>J. jaculus</i> and <i>R. norvegicus</i>
Thermal stability observation	Positive selection slightly enhanced Ucp1-ATP complex thermal stability in HETs	No notable impact of positive selection on Sirt1 thermal stability, though species-specific adaptations observed in NAD complex

Sirt1 is the Ucp1 expression regulator. It is activated by NAD (Zhao *et al.*, 2013) and was found to be positively selected in several species across different thermoregulatory groups, including daily heterotherms (*H. glaber*, *J. jaculus*), hibernators (*M. auratus*, *U. parryii*) and homeotherms (*R. norvegicus*, *C. griseus*, *C. porcellus*, *M. spicilegus*; Figure 13). We found that some of the 16 NAD-binding sites were absent in the studied rodents, with no clear pattern correlating the presence or absence of these sites with thermoregulatory strategy or phylogeny (Figure 18, Attachment 6). This suggests that species-specific adaptations may drive the absence or modification of these binding sites (Table 12). The RMSD values for Sirt1-NAD complexes were generally low (≤ 1) across all species, indicating tight structural alignment and that Sirt1-NAD binding is well-conserved across species, regardless of thermoregulatory strategy. All Sirt1-NAD complexes exhibited negative binding energies (ΔG), indicating favourable spontaneous NAD binding to Sirt1 ($\bar{x}$: -7.84 kcal/mol, range: -7.42 to -8.10 kcal/mol). No differences in binding energy variation were found across thermoregulatory groups, suggesting that the stability of the Sirt1-NAD complexes remain robust across thermoregulatory groups (Attachment 8). Protein denaturation across species at both 27°C and 37°C was higher in heterothermic species compared to homeotherms and hibernators (Table 12). Positively selected wild proteins exhibited higher denaturation than non-selected wild types, with ancestral and wild type positively selected forms showing similar differences in denaturation, suggesting that positive selection did not largely affect the thermal stability of Sirt1-NAD complexes (Table 12). Melting temperature simulations at 27 and 37°C revealed similar values across all species, generally low ($< 1^\circ\text{C}$), except for *J. jaculus* and *R. norvegicus* (Table 12, Attachment 8). This might indicate that Sirt1 function may be temperature-independent or adapted to species-specific thermoregulatory requirements and support the notion that Sirt1-NAD complexes are structurally adaptable to various temperature regimes, with specific adaptations in heterothermic species.

In summary, our analysis found no marked structural changes exclusive to homeothermic or hibernator species, nor were positively selected sites in conserved protein regions unique to any group; rather, some were shared between two groups. Daily heterotherms had amino acid substitutions in key thermoregulatory proteins, particularly Ucp1 and Sirt1 (which was positively selected also across groups). The substitutions in Ucp1 may influence ATP binding and protein thermal stability, supporting a broader temperature range, or marginally enhanced stability under fluctuating temperatures. In daily heterotherms positively selected Sirt1 sites may contribute to species-specific Sirt1-NAD adaptations. However, these changes do not significantly affect Sirt1's thermal stability or its ability to function across a wide temperature range, suggesting that Sirt1's role in thermoregulation involves more subtle adaptations rather than broad thermally driven changes.

Faster evolution of thermoregulatory networks in daily heterotherms

To further explore potential functional consequences of positively selected changes in thermoregulatory proteins, we examined their protein-protein interaction (PPI) network. Using the known Human proteome (comprising 6111 more interactors than mouse (11571); EvoPPI), we created a representative PPI network, with the 34 positively selected proteins as central nodes, focusing on their integration within broader networks.

We hypothesized that proteins positively selected in more flexible thermoregulators (daily heterotherms and hibernators) are more strongly integrated into the PPI network compared to proteins positively selected in homeotherms. Daily heterotherms and hibernators usually inhabit areas where they face fluctuating environmental and energy demands, likely requiring greater coordination across molecular pathways for managing such dynamic thermoregulation (Bastos *et al.* 2021).

The PPI network comprises a total of 164722 interactions, including 1799 first-degree and 162923 second-degree interactions involving the 34 thermoregulatory proteins with signs of selection (Figure 19).

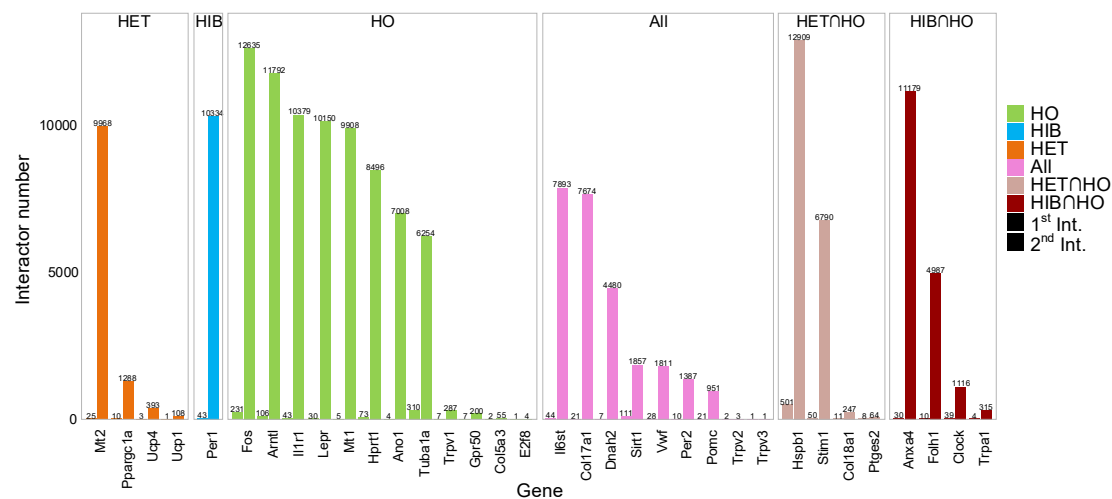


Figure 19 - Number of 1st and 2nd-degree Human protein-protein interactions in our 34 positively selected genes across thermoregulation strategy. Bar graph showing the number of positive selected genes and its first- and second-degree interactors number across each thermoregulation strategy.

These encompass both direct partners and extended connections within the full EvoPPI dataset. Due to shared interactors across thermoregulatory proteins, a single network is formed, structured into 14 total modules, that collectively include all thermoregulatory proteins. These 34 proteins are concentrated into 5 modules, with each containing at least two positively selected thermoregulatory proteins, which in turn are connected to the remaining 9 modules via shared interactors. Notably, the two proteins of module 5, E2f8 and Col5a3, were exclusively under positive selection in homeothermic species. Within each thermoregulation group, the proteins with the highest number of 1st-degree interactors were: Mt2 (25) in daily heterotherms, Per1 (43) in hibernators and Tuba1a (310) in homeotherms. In the 2nd-degree interactors those were: Mt2 (9968) in daily heterotherms, Per1 (10334) in hibernators and Fos (12635) in homeotherms. We compared the thermoregulation groups regarding the number of first- and second-degree interactors their positively selected proteins have. To account for differences in species number across thermoregulation strategies, the number of interactors per species were normalized. Hibernators had the highest number of interaction partners per selected proteins in both 1st- (direct) and 2nd-degree (indirect) connections, having 10.75 and 2583.5 interactors per selected species and per all group species, respectively. These

were followed by daily heterotherms, considering indirect interactions (1.95 and 587.85 1st and 2nd, respectively) and by homeotherms (5.69 and 535.88, 1st and 2nd, respectively). This pattern suggests that proteins under selection in hibernating and heterothermic species tend to be more network-integrated interacting with many other proteins while those under selection in homeotherms are more peripherally connected within the network.

While the proteins positively selected in daily heterothermic species have more neighbours (direct protein interactors) than homeotherms, their neighbours tend to be unique and not shared with other thermoregulatory proteins. For instance, *Ucp1*, which was positively selected in two daily heterothermic species, has a total of 109 1st or 2nd-degree neighbours but shares relatively few neighbours (26) with other thermoregulatory proteins. In contrast, proteins from the other groups often share more interactions. For instance, *Sirt1*, which was positively selected in most species has 1968 1st or 2nd-degree neighbours but has more than three times more neighbours than *Ucp1* in common with other thermoregulatory proteins (84). To understand which functions were influenced by *Ucp1* and *Sirt1*, we performed GO and KEGG pathway enrichment on their interaction partners. For *Ucp1*, GO enrichment highlights mitochondrial organization, protein localization to mitochondria and protein transmembrane transporter activity, emphasizing mitochondrial dynamics and molecular transport. Enriched KEGG pathways include mitophagy and autophagy in animals, nucleocytoplasmic transport and the NOD-like receptor signalling pathway, emphasizing roles in cellular degradation, transport and immune signalling. These findings suggest a broader role for *Ucp1* in detoxification and stress defence, potentially supporting adaptive responses to temperature fluctuations for heterothermic species in harsh environments. While *Ucp1* shares relatively few interactors with other thermoregulatory proteins, its own interactome reveals links to key cellular processes, including metabolic and stress-related pathways. Despite not being strongly interconnected within the core thermoregulatory module, its involvement supports an adaptive mechanism that, in heterothermic species, likely coordinating metabolic and stress responses to enable flexible thermoregulatory states (Gaudry *et al.*, 2018). Through its neighbours, *Ucp1* may help stabilize cellular functions during thermal changes, enabling efficient metabolic shifts and stress protection critical for heterothermic adaptation. For *Sirt1*, enriched GO terms relate to ribosome biogenesis, rRNA processing and ubiquitin-protein transferase activity. In addition, enriched KEGG pathways include ATP-dependent chromatin remodelling, RNA degradation and ubiquitin-mediated proteolysis, emphasizing roles in RNA metabolism, chromatin regulation, and protein degradation. These findings suggest that *Sirt1* plays a crucial role in maintaining cellular integrity and metabolic flexibility through several cellular processes that can be essential for adaptation to temperature fluctuations.

To summarize, we observed that the proteins under positive selection in hibernators directly exhibited 5.5 and 1.9 times greater inter-protein connectivity compared to daily heterotherms and homeotherms. Yet overall, the higher connectivity of positively selected protein-coding genes in hibernators and daily heterotherms suggests greater potential for integration into diverse cellular pathways. While this does not necessarily

imply increased pleiotropy, it may reflect a broader functional reach or regulatory complexity in their interactomes. This enhanced network integration could support the physiological flexibility needed for hibernation. Notably, hibernators showed high connectivity in both direct (1st-degree) and indirect (2nd-degree) interactions, while daily heterotherms had fewer direct connections but more indirect ones than homeotherms, suggesting distinct strategies where hibernators rely on broad integration, daily heterotherms on flexible indirect control, and homeotherms on more specialized, peripheral roles.

4.4. Discussion

Conservation of thermoregulatory genes among rodents

Our analysis of 54 thermoregulatory genes across 21 rodent species reveals a shared genetic framework underpinning diverse strategies, supporting the idea that thermoregulation is an ancient and adaptable trait (Grigg *et al.*, 2022; Koteja, 2004). The conservation of most genes among species exhibiting homeothermy, daily heterothermy, and hibernation suggests these strategies likely evolved independently multiple times (Boyles *et al.*, 2013), driven by gene expression/function shifts rather than new genes. Many thermoregulatory genes have dual roles in both cellular processes (e.g., cell cycle; Joshi *et al.*, 2022) and physiological adaptation. Phylogenetic analyses highlight the plasticity of thermoregulation, with strategy-switching possible within short evolutionary periods. While most genes are conserved, *Gpr50* and *Myo15a* show strategy- or clade-specific roles. *Gpr50*, found only in Muroidea, is linked to fasting-induced downregulation (Bechtold *et al.*, 2012; Ivanova *et al.*, 2008) and melatonin receptor modulation (Levoye *et al.*, 2006). Its distinct expression in torpor vs. hibernation (Gautier *et al.*, 2018) suggest functional divergence. *Myo15a*, mostly in the *Mus* genus, is associated with daily temperature downregulation in lab mice and Djungarian hamsters (Hudson & Scott, 1979; Cubuk *et al.*, 2017); its absence in hibernators suggests alternative adaptations for sensory function, with trade-offs in hearing/balance (Kanzaki *et al.*, 2006). Similarly, *Il1r1* is absent in hibernators, potentially avoiding neuroinflammation during torpor (Mueller *et al.*, 2014). Despite some missing orthologs (possibly due to genome quality), 81.5% of genes were present in at least 18 species, supporting the idea of a robust, conserved toolkit. Strategy-specific adaptations (e.g., *Gpr50*, *Myo15a*) likely enable fine-tuning of thermoregulatory responses. Future studies should focus on functional validation and regulatory mechanisms of such genes. Comparative transcriptomics and epigenomics across clades and thermoregulatory types, integrated with environmental data, will further elucidate these dynamics.

In summary, thermoregulation in rodents is supported by conserved genes, with implications for evolutionary biology and ecology.

Significance of positive selection among daily heterotherms

We found widespread positive selection in 35 of 54 thermoregulatory genes across 21 rodent species, emphasizing adaptive pressures on thermoregulation. Every species had at least one selected gene. Daily heterotherms showed the highest average of

positively selected sites (40.60), followed by homeotherms (37.91) and hibernators (31.25), supporting the view that temperature variability drives stronger selection. Although most positively selected genes are shared across strategies, site-specific differences suggest species-specific adaptation. *Ucp1*, involved in non-shivering thermogenesis, showed selection in only two heterothermic species, highlighting species-specific environmental influences rather than universal patterns. Notably, 1.8% of thermoregulatory genes showed signs of selection, a value within the general 1–3% range observed in rodents, reflecting rodents' unique thermal challenges. Similar patterns are seen in cold-adapted mammals like bats (Zu *et al.*, 2018). Evolutionary changes seem to favour modifying existing genes rather than introducing new ones. Slightly elevated adaptive changes in daily heterotherms reinforce the role of selective pressure in shaping thermoregulation. Future work should functionally characterize selected sites and integrate genomic, transcriptomic, and environmental data to clarify their roles.

In summary, thermoregulation evolves through a balance of shared and species-specific genetic adaptations, allowing rodents to thrive in diverse conditions.

Implications of functional changes in thermoregulatory Proteins

We examined how positive selection affects protein function and stability in daily heterotherms, focusing on *Ucp1* and *Sirt1*. Most positively selected changes (417 sites in 20 genes) were in non-conserved regions, likely with minimal impact. However, 10 genes had selected sites in conserved regions, possibly altering function. *Sirt1* and *Per2* had multiple such sites, consistent with findings that conserved regions harbour critical residues (Ruf & Geiser, 2015; Pancotti *et al.*, 2022). In daily heterotherms (*H. glaber*, *J. jaculus*), *Ucp1*'s selected residues affect ATP-binding, potentially improving stability at fluctuating temperatures, aligning with its role in non-shivering thermogenesis (Riley *et al.*, 2016; Grigg *et al.*, 2022). *Ucp1* proteins in daily heterotherms showed higher melting temperatures and lower denaturation at 37°C comparing with ancestral/non-selected proteins, indicating selection for thermoregulatory flexibility. *Sirt1*, which regulates *Ucp1* via NAD binding, showed positive selection across all strategies, with consistent binding energy but slightly reduced stability in daily heterotherms, suggesting a regulatory rather than structural role (Zhao *et al.*, 2013; Pancotti *et al.*, 2022). The ΔT_m was smaller in *Sirt1* than *Ucp1*, supporting its function across variable temperatures. Overall, daily heterotherms show distinct protein adaptations. Positively selected changes in *Ucp1* and *Sirt1* enhance protein function under thermal fluctuation, aligning with findings on physiological flexibility (Ruf & Geiser, 2015; Nowack *et al.*, 2020). However, the presence of shared selection signatures across thermal strategies, along with the absence of strong $\Delta\Delta G$ shifts in protein stability, points to more general adaptive pressures not restricted to daily heterothermy. Limitations of this analysis include reliance on *in silico* predictions and a limited protein and species set. Future work should include *in vitro* validation and explore additional genes like *Per2*.

In summary, functional changes in key proteins, notably *Ucp1* and *Sirt1*, may be supporting flexibility in daily heterotherms and advancing our understanding of molecular thermoregulation.

Thermoregulatory network evolution in flexible thermoregulators

We investigated how thermoregulatory proteins have evolved across mammals with different thermoregulatory strategies, focusing on 34 proteins under positive selection. By integrating protein-protein interaction (PPI) data, we assessed whether evolutionary changes support flexible thermoregulation under environmental challenges. Our analysis revealed that hibernators had the highest number of second-degree interactors, followed by daily heterotherms and homeotherms, suggesting that network complexity scales with thermoregulatory flexibility. Daily heterotherms, while having more first-degree interactors than homeotherms, showed less overlap in interactions, indicating more species-specific, possibly adaptive, PPI patterns. For example, Ucp1 had many interactors but few shared with other thermoregulatory proteins, whereas Sirt1 showed more conserved interactions. Functional enrichment revealed distinct roles: Ucp1 was associated with mitochondrial dynamics and stress responses, while Sirt1 was linked to chromatin remodelling and RNA degradation, highlighting their broader roles beyond thermogenesis.

Overall, our findings suggest that flexible thermoregulators evolved their network rewiring that expands functional integration. These adaptations may enhance pleiotropy, resilience, and versatility under thermal and energetic stress. Further experimental work is needed to validate these molecular strategies across thermoregulatory phenotypes.

4.5. Conclusion

Our results showed generally universal molecular mechanisms of thermoregulation across studied rodents representing classical thermoregulatory strategies of daily heterotherms, hibernators and homeotherms. In particular, we found that: 1) most genes associated with thermoregulation are shared across species; 2) many positively selected genes were shared among three thermoregulation strategies, with few unique to a single strategy; and 3) the protein structure of Ucp1 (which showed signs of selection only in daily heterotherms) and Sirt1 (which showed signs of selection in all strategies), showed only minor potentially functional differences among thermoregulation strategies. Partially supporting the hypothesis that heterothermy, but not strict homeothermy, is associated with a more flexible thermoregulatory molecular machinery we found that, 4) daily heterothermic rodents showed some changes in positively selected amino acid substitutions in thermoregulation-associated proteins. Particularly, changes in Ucp1 marginally increased the thermal stability of its protein complexes. Moreover, 5) flexible thermoregulators such as hibernators, had their selected proteins integrated into a broader thermoregulation protein interaction network, showing a more complex interactome than homeotherms. The clear limitation of this work stems from a small and unbalanced number of species among thermoregulatory strategies, limited by the available genomic data. Further, protein-protein interaction data is only available for human and mouse proteomes and not for non-model species, and thus our network inference was based solely on these two networks rather than on species-specific protein interaction information. Although this approach introduces a limitation, it allowed us to leverage the extensive and well-documented human and mouse interactomes, which are

often conserved across species and can offer valuable insights into broad evolutionary trends. Notwithstanding, our results provide the first evidence to suggest that changes in protein interaction networks, rather than changes within a single protein, are what drive evolution of flexible thermoregulatory strategies. Therefore, focusing solely on a few selected functional genes, rather than considering their broader interactome, may provide a limited perspective on the evolutionary dynamics of complex traits such as thermoregulation. Our findings also support the idea that classifying mammals into discrete categories, such as hibernators, daily heterotherms, and homeotherms, may oversimplify what is likely a continuum of adaptive strategies and molecular regulation (Boyles et al., 2013) and that adaptive lifestyles, and their underpinning molecular regulation, may be of gradual and quantitative nature, rather than dichotomous and binary.

Chapter 5. Co-expression of cognitive and metabolic network pathways across heterothermic levels in desert rodents

5.1. Introduction

Certain species rely on phenotypic plasticity to adapt to environmental fluctuations. Heterothermy, a physiological strategy of some endotherms, enables modulation of metabolic rates and body temperature in response to environmental changes (Ruf *et al.* 2012; Ruf and Geiser 2015; Landes *et al.* 2020). By transitioning between metabolically active and energy-conserving states (e.g., torpor; IUPS Thermal Commission 2003; Boyles *et al.* 2020), heterothermic species downregulate metabolism, cardiovascular activity and immune functions to maintain energy balance while under resource constraints (Carey *et al.* 2003; Klug and Brigham 2015). While the role of heterothermy in reducing energy consumption is well established, particularities regarding its molecular regulation remain underexplored (Dausmann 2014). By modulating metabolism and energy allocation, heterothermy may enhance resilience to thermal and resource variability, with effects extending beyond metabolism to influence cognitive processes (Ruf and Geiser 2015; Geiser 2020; Giroud *et al.* 2021). Metabolic shifts during heterothermy are associated with changes in lipid metabolism, oxidative phosphorylation and mitochondrial efficiency, processes controlled by gene expression (Carey *et al.* 2003; Ruf *et al.* 2012; Klug & Brigham 2015; Boyles *et al.* 2020). While some molecular mechanisms are characterized, the extent to which heterothermy drives coordinated changes in gene networks underlying metabolism and cognition remains unclear (Luu *et al.* 2016; Crippen *et al.* 2024). Temporary reductions in neural activity during torpor may impair perception, learning and responsiveness to threats, suggesting a potential trade-off between energy conservation and cognitive stability (Jastroch *et al.* 2016; Hitrec *et al.* 2019; Nowack *et al.* 2019). We propose that this trade-off reflects a fundamental biological balance between network flexibility and stability: flexible networks may promote rapid physiological adaptation at the cost of reduced robustness, while stable networks may safeguard critical functions such as cognition but limit the potential for plastic responses. Recent evidence (chapter 4) suggests that heterothermic species exhibit smaller yet more interconnected gene co-expression networks regulating metabolism and cognition, facilitating efficient physiological transitions during thermal stress (Nowack and Dausmann 2015; Verble *et al.* 2024). Therefore, understanding how daily heterothermy influences metabolic and cognitive regulation at the gene network level is critical for elucidating how these species adapt to fluctuating environmental conditions (Sieck 2002; Cubuk *et al.* 2017; chapter 4). Species with varying degrees of heterothermy are expected to exhibit distinct gene expression patterns regulating metabolism and cognition, facilitating adaptive responses to environmental fluctuations (Luu *et al.* 2016; Verble *et al.* 2024). Previous studies suggest that heterothermic species tend to show interconnected gene networks (Crippen *et al.* 2024), despite involving fewer genes, highlighting their potential to cope with environmental stressors through plastic adjustments (Nowack and Dausmann 2015). However, most studies have focused on

temperature-driven gene expression changes at an individual-gene level, rather than investigating network-wide adaptations that may contribute to phenotypic plasticity in response to environmental stress (Mitchell *et al.* 2002; Hitrec *et al.* 2019; Crippen *et al.* 2024).

We hypothesize that species with greater daily body temperature fluctuations have more flexible gene co-expression networks regulating metabolism- and cognition, enabling plastic adjustments under thermal stress. In contrast, species with lower daily body temperature fluctuations have more robust, but less flexible networks. To test this, we analyse three desert rodent species with varying degrees of daily heterothermy, investigating how their gene networks orchestrate metabolism and cognition respond to fluctuating conditions. To test this biological trade-off, we compare the network (stable/flexible - α/γ nodes) organization of three rodent species exhibiting varying levels of daily heterothermy.

Hypothesis: Species with greater daily body temperature fluctuations have flexible gene co-expression networks regulating metabolism and cognition, enabling plasticity under thermal stress. In contrast, species with lower daily body temperature fluctuations prioritize have more stable, but less flexible networks.

Research questions:

- Q1 - To what extent does intra-individual variation in body temperature between active (night) and inactive (day) phases of circadian rhythms quantify the levels of downregulation among species?
- Q2 - Do day/night differential gene expressions of metabolic and neural/cognitive functions vary according to the species' thermoregulation patterns?
- Q3 - Do the sizes and connectivity of metabolic and neural/cognitive networks vary between active and inactive phases, and among species' thermoregulation patterns?
- Q4 - Are there trade-offs between stability (shared nodes) and flexibility (rewiring) of the networks, and is higher rewiring observed in daily heterotherms?
- Q5 - Are genes responsible for network rewiring specifically associated with metabolic and cognitive regulation?

5.2. Materials and methods

Body temperature measurements

Rectal body temperature (T_{body}) measurements were taken during field conditions using a BIO-RET-3 probe (HANNA Instruments) for all three species (*J. jaculus*, *J. hirtipes*, *G. gerbillus*). Measurements were performed both at night (21:00h-6:00h) and during the day (6:30h-15:00h). Temperature readings were obtained within 2 minutes per animal. In total, 30 *J. jaculus*, 22 *J. hirtipes*, and 64 *G. gerbillus* were measured, out of which 10, 10, and 20 individuals, respectively, were later subjected to RNA sequencing (Attachment 9).

Sample collection and sequencing

Animal capture and handling were conducted under local permits, in compliance with European Directive 2010/63/EU. Tissue samples were collected from *J. hirtipes* (10 specimens: 3 females from Morocco, 7 males from Morocco and Mauritania), *J. jaculus* (11 specimens: 5 females and 6 males from Mauritania), and *G. gerbillus* (20 specimens: 9 females and 11 males from Morocco). We sampled animals during their active night periods (homeothermic physiology) and inactive day periods (daily heterothermic physiology). Dissected tissues included the hippocampus, amygdala, liver, and dorsal skin for transcriptome sequencing. Genomic DNA was extracted from ear tissue using a chloroform-based method, and Illumina HiSeq2500 sequencing was employed for both transcriptome and whole-genome sequencing, following standard paired-end sequencing protocols (Meyer and Kircher 2010).

Genome mapping and RNAseq data processing

Paired-end RNAseq reads for *J. hirtipes*, *J. jaculus*, and *G. gerbillus* were aligned using HISAT2, with *J. jaculus* reads mapped to the mJacJac1.mat.Y.cur reference genome and *G. gerbillus* to *M. unguiculatus* (MunDraft-v1.0), respectively. Before mapping, FastQC analysis was performed to ensure the quality of the paired-end reads. The overall alignment rates averaged 96.13%, 80.32%, and 46.74% for *J. jaculus*, *J. hirtipes*, and *G. gerbillus*, respectively. Although the alignment mapping rate for *G. gerbillus* appeared low, the featureCounts analysis revealed significant coverage of the “protein coding” section from the m_unguiculatus.gff file. Mapped reads were counted using featureCounts based on the respective GFF annotation files (Liao *et al.* 2014). All tissues from each species were included in the gene expression analysis pipeline.

Cognition and metabolism associated gene lists

AmiGO 2 and Human phenotype ontology databases (<https://amigo.geneontology.org/amigo/search/ontology> and <https://hpo.jax.org/>) were used to collect cognition (3169) and metabolism (2133) associated genes, by searching for learning, memory, cognitive function, intellectual/cognitive/learning disability, cognitive regression, abnormal cognitive process, global cognitive delay and cognitive impairment; and metabolism, energy homeostasis, thermogenesis, lipid metabolism. These Human genes were posteriorly converted to *Mus musculus* orthologues to create our gene lists. From these lists, in the original 12242 shared mapped genes across the three species we found 2375 shared genes (reads > 0) mapped for cognition and 1528 shared genes mapped for metabolism.

Gene expression patterns

Differential gene expression (DGE) analysis was conducted using DESeq2 (Love *et al.* 2014), with samples categorized as either control (night samples) or torpid (day samples). Analyses were performed for various organs and conditions: (A) across hippocampus and liver tissues for *J. jaculus*, *J. hirtipes* and *G. gerbillus* with control for species, condition, sample type, tissue, and sex;

```
jj_liver_dds=DESeqDataSetFromMatrix(countData = jj_liver_readcounts, colData =
jj_liver_coldata_jac, design = ~ Condition+Sex). Genes with more than 0 reads, Padj <
0.05, were considered differentially expressed (Table S3).
```

Gene ontology (GO) and pathway (KEGG) enrichment analysis

GO enrichment analysis was performed in all significant γ nodes, using *Mus musculus* orthologues for those genes in all our three species. Relevant GO terms were selected ($P_{adj} < 0.05$; Table S10). Overrepresentation tests for biological processes (BP) and molecular functions (MF) were conducted using “bitr” from clusterProfiler and the org.Mm.eg.db package (Carlson 2019), considering species, tissue and genes (metabolic or cognitive). KEGG pathway enrichment analysis followed a similar approach, using clusterProfiler and overrepresentation tests via org.Mm.eg.db. Pathways relevant to cognition and metabolism ($p\text{-value} < 0.05$) were selected from "Cellular Processes," "Genetic Information Processing," "Environmental Information Processing," and "Human Diseases," resulting in 39 cognition-related and 51 metabolism-related terms (Table S11).

Weighted and co-expression network gene analysis

Gene co-expression networks were constructed for each tissue using the wTO method (Gysi *et al.*, 2018) with Pearson correlations and bootstrapped using 1000 replicates, in both night and day readcounts, that were contrasted either with cognition or metabolism-associated genes. Co-expression networks were compared across species and tissues using CoDiNA (Gysi *et al.* 2020), categorizing gene interactions into common, different, and specific categories. For these classified gene interactions, both gamma and beta nodes, that showed significant differential expression values and that belong to the best 5% wTO nodes with highest link number were posteriorly analysed as candidate genes.

Network flexibility testing:

A. Comparison of network size and connectivity

The Weighted Topological Overlap (wTO) networks for each species and tissue type were filtered using a threshold of $p_{value_sig} < 0.01$. The number of unique nodes, edges, and network density were computed for each network and node connectivity was assessed by counting the number of direct connections per node. To compare network properties across conditions (night and day), the number of nodes, edges, and network density ($Density = E/(N-1)/2$, E = number of edges and N = number of nodes) were quantified separately for day and night conditions. Differences in connectivity between conditions were computed at both the network and node levels. The average connectivity difference per node was calculated by grouping data by network and computing the mean difference between day and night values.

B. Statistical analysis of network properties

A two-way ANOVA was performed to assess the effects of species, tissue type, and condition (day/night) on network size (number of nodes) and connectivity. Post hoc pairwise comparisons were conducted using Tukey's Honest Significant Difference (TukeyHSD) test. To assess wTO network rewiring, the difference in pvalue_sig values (<0.01) between day and night conditions (ΔwTO_sign) was calculated for each common gene pair (Node.1Node.2). The proportion of positively ($\Delta wTO_sign > 0$) and negatively ($\Delta wTO_sign < 0$) rewired edges was computed across species, tissues, and gene categories.

C. Node classification and plasticity tests in CoDiNA networks

To assess functional network rewiring, CoDiNA package was used classify nodes into stability categories: conserved (α), strongly rewired (γ), and condition-specific (β). Nodes with a rewiring score (Φ_tilde) greater than 1 were classified as γ . The proportions of α , γ , and β nodes were quantified across networks and the statistical differences in the proportion of γ nodes were assessed using ANOVA analysis, with species, tissue type, and gene category as independent factors. Posterior TukeyHSD was applied for post hoc comparisons. Besides, to evaluate species-specific differences in rewiring, the proportion of γ nodes was compared across species and tissue types. An ordinal logistic regression model was employed to assess the effect of heterothermy on rewiring dynamics, here species were categorically classified as "high-low-minimal" corresponding to *G. gerbillus*, *J. jaculus* and *J. hirtipes*. And finally, the stability and flexibility of networks were further evaluated by analysing the α to γ ratio and its correlation with network size and density. This approach provided insights into the balance between conserved and rewired components across species and conditions. All analyses were performed in R, using packages such as "dplyr" for data wrangling and "CoDiNA" for differential network analysis. Processed datasets were exported as CSV files for further examination and visualization.

5.3. Results

Higher level of temperature downregulation in *G. gerbillus* than in *Jaculus* species

To understand if species differ in their levels of daily temperature downregulation, a total of 116 body temperature measurements were conducted for 30 *J. jaculus*, 22 *J. hirtipes*, and 64 *G. gerbillus*. Of these, some individuals from each species were later selected for RNA sequencing (10 *J. jaculus*, 10 *J. hirtipes*, and 20 *G. gerbillus*; Figure 20, Figure 21, Attachment 9).

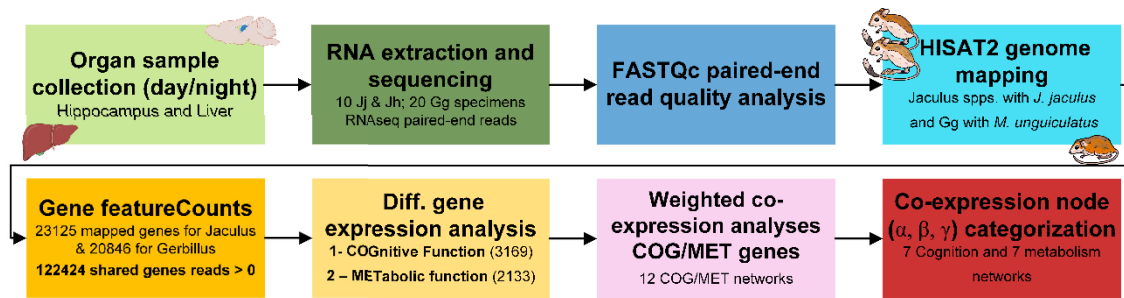


Figure 20 - Methodology overview. Flowchart summarizing the methodology processes and analyses conducted in this paper in *J. jaculus*, *J. hirtipes* and *G. gerbillus*. Bold text represents main methodology steps, while normal text denotes corresponding results.

The average body temperatures recorded during the night for each species were 37.0°C for *G. gerbillus*, 38.1°C for *J. jaculus* and 38.9°C for *J. hirtipes* (Figure 20, Figure 21, Attachment 9).

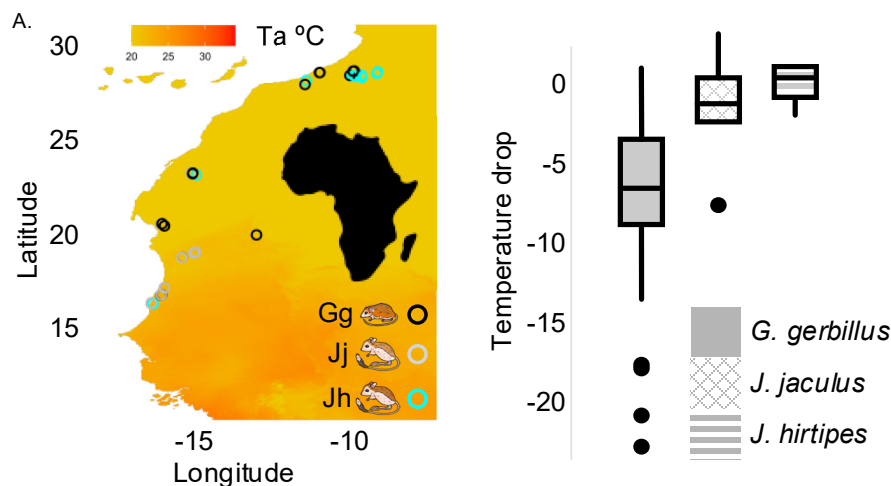


Figure 21 - A. Species samples geographical dispersal. Colour gradient from red to yellow represents average ambient temperature, black circles for collected *G. gerbillus* samples, cyan circles for *J. hirtipes* samples and grey circles for *J. jaculus* samples. B. Dispersion of the variation of body temperature ($\Delta T_{\text{Body}} = T_{\text{Body day}} - T_{\text{Body night}}$) across all field analysed specimens 64, 30 and 22 (sequenced plus non-sequenced) for *G. gerbillus*, *J. jaculus* and *J. hirtipes*.

In contrast, the average daytime body temperatures were 30.2°C for *G. gerbillus*, 36.5°C for *J. jaculus* and 37.9°C for *J. hirtipes*. The levels of temperature downregulation observed were higher for *G. gerbillus* (-7.19°C; max: -21.9, min: 0.2°C), followed by *J. jaculus* (-1.76°C; max: -7.8, min: 2.4°C), and *J. hirtipes* (-0.72°C; max: -2.7, min: 0.3°C). These results indicate significant differences in thermoregulation strategies among the three species. Notably, *J. hirtipes* exhibited the least body temperature downregulation, suggesting a more stable thermoregulatory capacity during day and night cycles. In

contrast, *G. gerbillus* displayed a more pronounced temperature downregulation (Ghyrs *et al.* 2021).

On average, females exhibited a higher downregulation (by 1.13°C) than males across all species. More specifically, females showed greater downregulation in *G. gerbillus* (-1.95°C), *J. jaculus* (-0.38°C), and *J. hirtipes* (-1.07°C) compared to males. Night temperatures were, on average, higher in females than males in both *Jaculus* species, while the opposite was observed in *G. gerbillus*, where males had higher temperatures than females. Conversely, daytime temperatures were higher in males than females in *G. gerbillus* and *J. hirtipes*, but the opposite was observed in *J. jaculus*, where males had lower temperatures than females. These sex-related differences in temperature downregulation may reflect physiological variations in the daily rhythmicity of body temperature, possibly influenced by hormonal cycles (Refinetti R., 2020). Additionally, the ecological habits of each species could also play a role.

To address Q1, we quantified intra-individual variation in body temperature between active (night) and inactive (day) phases across the three studied species. Overall, the results reveal marked interspecific differences in daily thermal downregulation.

Hippocampal and hepatic distinct patterns of gene expression in response to thermal fluctuations

To understand if our species show distinct patterns of gene expression, a Differential Gene Expression (DGE) analysis was performed on the liver and hippocampus tissues, comparing night and day samples across all three species. Between night and day samples, for the liver, we identified a total of 1615 differentially expressed genes ($p_{adj} < 0.05$, DEGs; Figure 22, Table 13 and Attachment 10) in *G. gerbillus* 58 in *J. jaculus* and 22 in *J. hirtipes* (Table 13 and Attachment 10).

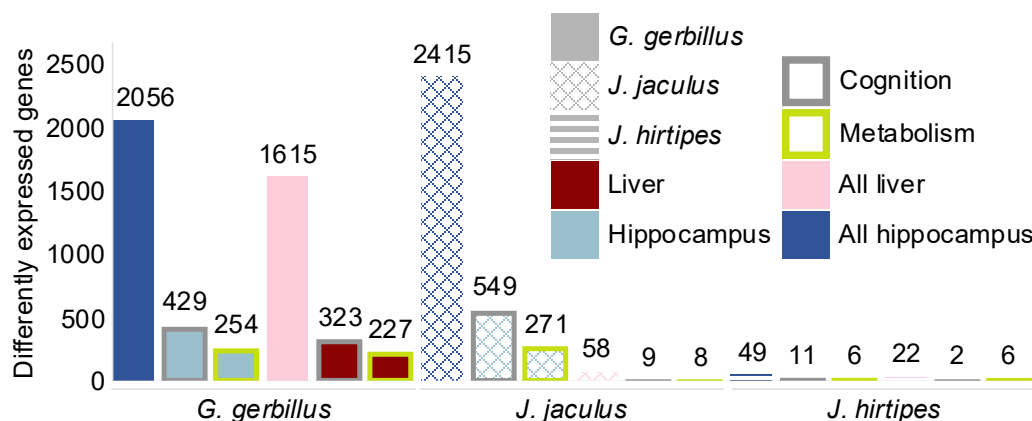


Figure 22- Complete organ differential gene expression across species and organs versus cognition and metabolic specific gene expression across species and organs between night and day samples. In dark blue, in hippocampus and in salmon, in liver. And percentage of those genes associated to metabolism or cognition in hippocampus, in light blue and in liver in dark red.

Table 13 - Differential gene expression results across species and tissues concerning cognition and metabolism-associated genes.

species	Tissue	All dif. expressed genes (padj < 0.05)	cognition DEGs (padj < 0.05)	metabolism DEGs (padj < 0.05)	% cognition genes	% metabolism genes
<i>G. gerbillus</i>	Hippocampus	2056	429	254	20.87	12.35
	Liver	1615	323	227	20	14.06
<i>J. jaculus</i>	Hippocampus	2415	549	271	22.73	11.22
	Liver	58	9	8	15.52	13.79
<i>J. hirtipes</i>	Hippocampus	49	11	6	22.45	12.24
	Liver	22	2	6	9.09	27.27

For the hippocampus, we identified a total of 2056 DEGs in *G. gerbillus*, 2415 in *J. jaculus* and 49 in *J. hirtipes*. To study cognition and metabolism, we did a literature review and compiled two comprehensive lists of genes associated with cognition, learning, cognitive/learning disabilities, and memory and metabolism, energy and thermogenesis, in *M. musculus*, totalling 3169 and 2133 genes, respectively. In our analysis of the differentially expressed genes, across night and day samples, for *G. gerbillus* we found 20% of the cognition-associated genes were significantly differentially expressed in the liver and 20.87% in the hippocampus. For *J. jaculus*, 15.52% in the liver and 22.73% in the hippocampus and for *J. hirtipes*, 9.09% in the liver and 22.45% in the hippocampus. The hippocampus percentages of DE cognition genes were similar in *Jaculus* species and slightly lower in *G. gerbillus*. The liver percentages of DE cognition genes were more scattered, having *J. hirtipes* the lowest values and *G. Gerbillus* the highest. Concerning the metabolism-associated genes, across night and day samples, for *G. gerbillus*, 14.06% in the liver and 12.35% in the hippocampus. for *J. jaculus* we found that 13.79% were significantly expressed in the liver and 11.22% in the hippocampus. For *J. hirtipes*, 27.27% in the liver and 12.24% in the hippocampus. The hippocampus percentages of DE metabolism genes were similar within the three species, having *J. jaculus* a slightly lower value.

Liver percentages of DE metabolism genes were more scattered, having *J. hirtipes* the highest values and *J. jaculus* the lowest. Our study aimed to investigate how species exhibiting daily heterothermy adjust gene expression in response to thermal and consequently energetic fluctuations caused by daily heterothermy, particularly focusing on the balance between energy conservation and brain function preservation. In the liver, the species demonstrated differing patterns of metabolism-associated gene expression. *G. gerbillus*, with the highest degree of heterothermy, showed 14.06% of metabolism-associated genes significantly differentially expressed. *J. jaculus*, exhibiting lower heterothermy, had a similar value of 13.79%, while *J. hirtipes*, the species with minimal heterothermy, showed the highest percentage at 27.27%. These findings suggest that, despite the higher percentage of DEGs metabolism-associated genes, *J. hirtipes* shows minimal overall gene expression changes between night and day, which may indicate that the small body temperature variation (Figure 22) has little effect on it, and other factors such as foraging and digestion, could contribute to the observed metabolic and

cognitive expression changes. Whereas *G. gerbillus* may rely more on maintaining metabolic homeostasis through its higher degree of heterothermy. In the hippocampus, the percentages of metabolism-associated genes were similar across species, *G. gerbillus* (12.35%), *J. jaculus* (11.22%), and *J. hirtipes* (12.24%). This indicates an overall consistent response to energy fluctuations at the brain level. Regarding cognition-related genes in the hippocampus, the expression levels were again similar, but *J. jaculus* exhibited the highest percentage of differentially expressed genes at 22.73%, followed by *J. hirtipes* (22.45%) and by *G. gerbillus* (20.87%). In the liver, the expression of cognition-related genes was notably different: *G. gerbillus* showed the highest percentage (20%), followed by *J. jaculus* (15.52%), and *J. hirtipes* had the lowest (9.09%). The adjustments to metabolism gene expression, particularly in the liver, highlight the active energy conservation mechanisms in *J. hirtipes*, while *G. gerbillus* seems to rely more on metabolic homeostasis through its pronounced heterothermy. At the same time, brain function is preserved across species, with cognitive function appearing to be prioritized in *J. jaculus*, a species with lower heterothermy, as evidenced by the higher percentage of cognition-related genes expressed in its hippocampus.

To address Q2, we examined whether day/night differential gene expression in metabolic and cognitive functions varied across species with different thermoregulatory profiles. *G. gerbillus*, the most heterothermic species, showed notable liver gene expression, but not the highest percentage of metabolic gene changes. *J. jaculus*, with moderate heterothermy, had the highest proportion of cognition-related expression in the hippocampus, suggesting a prioritisation of brain function. In contrast, *J. hirtipes*, with minimal heterothermy, showed the highest percentage of metabolic gene changes in the liver, implying that factors beyond body temperature, such as foraging or digestion, may influence expression. Overall, species exhibit distinct but partially overlapping transcriptomic strategies to balance energy and cognitive demands under thermal stress (Q2).

Cognitive and metabolic gene network connectivity reflects species' thermoregulatory flexibility

To understand whether cognition- and metabolism-associated gene networks differ in size and connectivity across species with different levels of daily temperature downregulation, for their weighted gene co-expression networks (wTO), we assessed the overall network size (nodes+edges) and connectivity (neighbours per gene) of gene networks regulating metabolism and cognition ($p_{val_sig} < 0.01$). Our analysis revealed no significant differences in network sizes across species and tissues and day-night time conditions. Yet, regarding associated genes, network size was significantly smaller for metabolism networks, compared with cognitive networks ($F = 7.379$, $p = 0.014$, Figure 23, Table S4). Our results revealed significant differences in networks connectivity across species, tissues, and night-day time conditions, in metabolism and cognitive networks (species: $F = 27.63$, $p = 1.02E-12$; tissue: $F = 20.24$, $p = 6.85E-06$; condition $F = 8.11$, $p = 0.0044$; Figure 24, Table 14, Tables 2, S5 and S6).

Genes with increased connectivity (18 genes for each tissue, in each network; Figure 24, Table 14) related to cognition in hippocampus, among all species, were primarily linked to glial-associated phenotypes, including neuromuscular abnormalities and peripheral nerve degeneration.

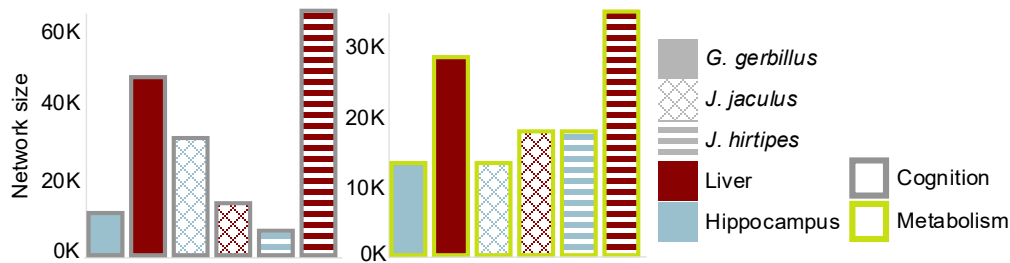


Figure 23 - Network size within liver and hippocampus tissues across species. Network size (number of edges plus number of nodes, pvalue < 0.01) across species. Hippocampus is here represented in blue and liver in dark red.

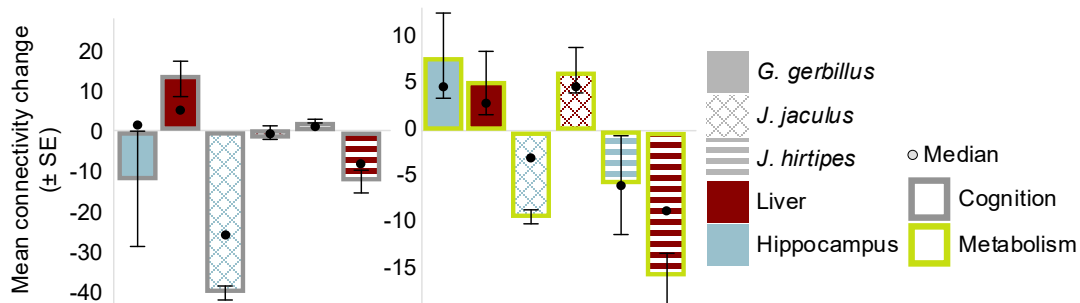


Figure 24 - Network connectivity change (between day and night) within liver and hippocampus tissues across species. Network size (number of edges plus number of nodes, pvalue < 0.01) across species. Hippocampus is here represented in blue and liver in dark red.

In liver, cognition-related genes with higher connectivity were enriched in processes related to developmental regulation, cranial nerve formation, transcriptional control and organogenesis. Metabolism-related genes with increased connectivity in hippocampus, were enriched for oxidative response, apoptosis regulation, immune and mesenchymal development, cellular signalling and communication, these genes taking part in several protein complexes involved in signalling integration and developmental regulation. In the liver, highly connected metabolism genes were enriched for pathways related thermogenesis (adaptive and cold induced), lipid transport and multicellular regulatory processes, which take part in several protein complexes involved in immune signalling and metabolic regulation.

Table 14 - Average node pairs connectivity changes and best positive (day enhanced) and negative (night enhanced) changing genes between night and day among genes, tissues and species, its mean and median.

Genes	Tissue	Species	Mean change	Median change	Best positive changing genes	Best negative changing genes	Mean change tissue	Mean change genes
Cognition	Hippocampus	<i>G. gerbillus</i>	-14.47	1	<i>Add3, Aldh18a1, Aptx</i>	<i>Foxe1, Golga2, Inpp5e</i>	-17.64	
		<i>J. jaculus</i>	-40.32	-26	<i>Brat1, Gle1, Rbl2</i>	<i>Fancg, Cog8, Golga2</i>		
		<i>J. hirtipes</i>	1.86	1	<i>Slc32a1, Dpm2, Ctsf</i>	<i>Capn1, Klc2, Nptx2</i>		
	Liver	<i>G. gerbillus</i>	14.20	4	<i>Sorcs3, Rorb, Mymk</i>	<i>Pax2, Foxe1, Irx5</i>	0.54	-8.55
		<i>J. jaculus</i>	-0.12	0	<i>Sct, Alx1, Hcrt</i>	<i>Phox2a, Otx2, Dlk1</i>		
		<i>J. hirtipes</i>	-12.45	-8	<i>Tmem270, Foxg1, Otx2</i>	<i>Foxe1, Mymk, Sct</i>		
Metabolism	Hippocampus	<i>G. gerbillus</i>	8.06	5	<i>Pitx2, Mettl21c, Tyr</i>	<i>Fabp2, Gpihbp1, Il1b</i>	-2.55	
		<i>J. jaculus</i>	-9.72	-3	<i>Pdcd4, Tcf7l2, G0s2</i>	<i>Mtmr2, Slc25a4, Ugcg</i>		
		<i>J. hirtipes</i>	-5.99	-6	<i>Pitx2, Cat, Stxbp1</i>	<i>Hand2, Abca2, Abl1</i>		
	Liver	<i>G. gerbillus</i>	5.31	3	<i>Avpr1b, Galr1, Oc90</i>	<i>Ikake, Mogat1, Pth2r</i>	-1.46	-2.01
		<i>J. jaculus</i>	6.45	5	<i>Sct, Avpr1b, Amer3</i>	<i>Nr5a1, Gpr3, Clps</i>		
		<i>J. hirtipes</i>	-16.14	-9	<i>Lta, Oxt, Rpe65</i>	<i>Nr5a1, Sct, Avpr1b</i>		

G. gerbillus, known for its high daily heterothermy (Ghyrs *et al.* 2022), overall shows flexible control in cognitive pathways (Figure 24), with night-enhanced connectivity in the hippocampus (-14.47, negative value means more night connectivity) and day-enhanced connectivity in the liver (14.2, positive value means more night connectivity). In metabolic pathways, connectivity is consistently day-enhanced across both tissues (8.06 and 5.31, respectively). *J. jaculus*, a species with lower daily heterothermy levels, shows strongly night-enhanced connectivity in cognitive control, especially in hippocampus (-40.32) and almost near neutral pattern in the liver (-0.12). In metabolic control, it shows night-enhanced connectivity in hippocampus (-9.72) and day-enhanced connectivity in the liver (6.45), reflecting moderate flexibility in regulatory coordination. *J. hirtipes*, characterized by minimal heterothermy levels, shows opposite control in cognitive pathways, with a day-enhanced connectivity on the hippocampus (1.86) and night-enhanced connectivity in the liver (-12.45). For metabolic control, it exhibits consistent night-enhanced connectivity (-5.99 and -16.14), indicative of a more stable and less flexible regulatory strategy. This trend of night-enhanced hippocampal connectivity aligns with the circadian modulation of memory, neurogenesis, and synaptic plasticity typically observed during resting periods in nocturnal animals (Norman F. Ruby *et al.* 2008; Gerstner and Yin 2010). Day-enhanced liver metabolic connectivity in highly heterothermic species (e.g., *G. gerbillus*) may reflect flexible responses to extreme daily body temperature downregulation, potentially involving metabolic preparation for nighttime activity.

To address Q3, we examined whether the size and connectivity of metabolic and cognitive gene co-expression networks differ between day and night phases and across species with varying degrees of thermoregulatory flexibility. Our results show that cognitive gene networks tend to be more connected at night (-8.55), potentially aligning with nocturnal memory consolidation or glial activity. In contrast, while metabolic

networks display more variable patterns of connectivity, possibly reflecting interspecific differences in thermogenic demands and circadian regulation shaped by species-specific thermophysiological strategies. These results support the hypothesis that greater thermoregulatory plasticity is associated with more adaptable gene network coordination, especially across functional domains like metabolism and cognition. Conversely, species with reduced thermoregulatory fluctuations appear to favour more rigid and consistent gene co-expression architectures, potentially ensuring homeostatic stability (Q3).

Heterothermy-driven network flexibility

To assess differences in weighted co-expression gene networks between day and night, we calculated variations in rewiring (ΔwTO_sign) for all node pairs ($pvalue < 0.01$). We then examined the proportion of positive rewiring across species, which reflects strengthened connections during the daytime body temperature downregulation (Figure 25, Attachment 12). Only *G. gerbillus* showed values for both positive and negative rewiring. For cognitive networks (~ 0.03 ; ~ 0.06 , positive/negative) and for metabolic networks (~ 0.15 ; ~ 0.04 , positive/negative).

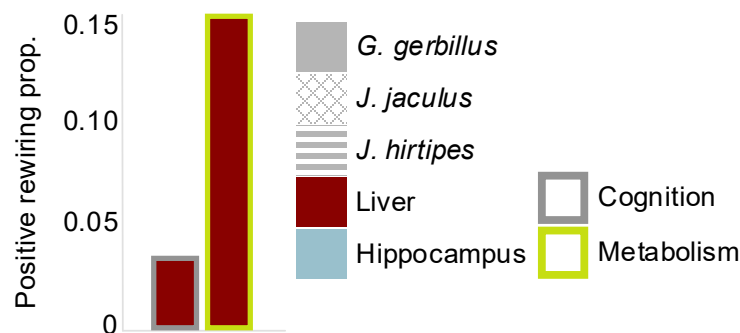


Figure 25 - Proportions of positive rewiring variation ($\Delta wTO_sign_prop > 0$, $pvalue < 0.01$). Hippocampus is here represented in blue and liver in dark red.

Next, we investigated trade-offs between stability and flexibility in metabolic and cognitive networks using a Co-expression Network Analysis (CoDina). This identified α (common), γ (specific/rewired), and β (sign-changing (+ $\rightleftharpoons$ -) nodes across species, tissues, and network types (Figure 26, Attachment 13).

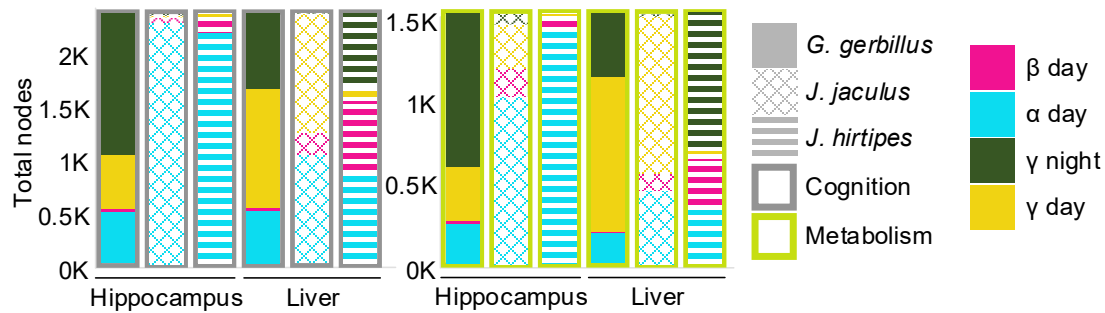


Figure 26- Total number and type of CoDina nodes within liver and hippocampus tissues across our species. Number and type of nodes found in each tissue on CoDina analysis across species. On the left, cognition associated results (grey outline) and on the right metabolism associated results (green outline).

We found a significant species effect in γ node proportions ($\text{Score_Phi_tilde}/\text{Score_internal} > 1$; $\text{cutoff.external} = 0$, $\text{cutoff.internal} = 1$). *J. hirtipes* ($p = 0.0004$) and *J. jaculus* ($p = 0.0019$) had significantly fewer of γ nodes than *G. gerbillus*. Tukey post hoc tests confirmed this pattern (*J. hirtipes* < *G. gerbillus*, $p = 0.0011$; *J. jaculus* < *G. gerbillus*, $p = 0.0047$), with no difference between *J. hirtipes* and *J. jaculus* ($p = 0.39$). The γ node proportions were also tissue-dependent, with the liver showing significantly higher values than the hippocampus ($p = 0.0063$). However, no significant differences were observed between metabolic and cognitive networks (TukeyHSD: $p = 0.22$). To test the stability-flexibility trade-offs, we correlated α (common, stable) and γ (rewired, flexible) node proportions, using a linear regression model with interaction terms ($\gamma \text{ proportion} \sim \alpha \text{ proportion} * \text{species}$, where $\alpha/\gamma < 0.5$ meant high flexibility, > 0.5 to 6 , medium and > 6 low flexibility). The model fit was strong ($R^2 = 0.991$, Adjusted $R^2 = 0.984$; $F(5,6) = 137.8$, $p = 4.18 \times 10^{-6}$) and results for this model showed significant main effect of α proportion ($F(1,6) = 642.2$, $p = 2.49 \times 10^{-7}$) and of species ($F(2,6) = 19.60$, $p = 0.0023$), with *J. hirtipes* showing a reduction in γ proportion of -0.571 [95% CI: -0.796 , -0.346] and *J. jaculus* of -0.443 [95% CI: -0.668 , -0.218], relative to *G. gerbillus*, indicating that both variables significantly influence γ proportion (flexibility). The interaction term showed only marginal effect ($F(2,6) = 3.90$, $p = 0.082$), suggesting potential species-specific differences in the slope of the relationship are small.

We quantified flexibility using the α/γ ratio (Table S9). *G. gerbillus* exhibited the highest flexibility in both metabolic and cognitive networks (α/γ ratio, metabolism: hippocampus = 0.20 , liver = 0.12 ; cognition: hippocampus = 0.28 , liver = 0.26). *J. jaculus* displays moderate flexibility (metabolism: hippocampus = 3.05 , liver = 0.46 ; cognition: hippocampus = 5.64 , liver = 0.90), while *J. hirtipes* shows low flexibility (metabolism: hippocampus = 64.36 , liver = 0.36 ; cognition: hippocampus = 88.04 , liver = 0.99). Ordinal logistic regression model was used to assess whether the proportion of γ (rewiring) predicts the degree of heterothermy, treated as an ordered categorical variable with three levels (“minimal”, “low”, “high”) corresponding to *J. hirtipes*, *J. jaculus* and *G. gerbillus*, respectively. The model revealed a significant positive association between γ proportion and heterothermy level ($\beta = 7.20 \pm 2.97$, $p = 0.015$), indicating that increased network rewiring is associated with greater heterothermy. Estimated thresholds separating

heterothermy categories were minimal|low = 1.94 and low|high = 4.73. These results suggest that species exhibiting high heterothermy, such as *G. gerbillus*, show enhanced rewiring.

In response to Q4, we investigated trade-offs between stability (shared network nodes) and flexibility (rewiring) in gene co-expression networks across species with varying levels of daily heterothermy. Our results clearly support the existence of such a trade-off. *G. gerbillus*, the species with the highest heterothermy, exhibited the most flexible gene co-expression networks, while *J. jaculus* showed intermediate flexibility, and *J. hirtipes*, which displayed minimal heterothermy, had the most stable but least flexible networks. This pattern aligns with species' thermoregulatory profiles. Notably, these networks were constructed from gene sets already enriched for metabolic and cognitive functions; therefore, the observed rewiring does not imply further functional enrichment but rather reflects dynamic changes in connectivity among genes within these predefined functional categories. Moreover, a strong negative correlation between network stability (α) and rewiring (γ) substantiates the predicted inverse relationship between robustness and plasticity. These findings validate the hypothesis that higher heterothermy is associated with greater gene network adaptability in both metabolic and cognitive functions (Q4).

Rewiring genes reflect a hippocampal heterothermy-associated gradient in transcriptional regulation

Testing whether rewiring genes are associated with metabolic and cognitive regulation revealed a hippocampal heterothermy-linked gradient in transcriptional activity. To understand if genes driving network rewiring were involved in metabolic and cognitive regulation, on the liver and hippocampus, respectively, we performed both GO and KEGG enrichment analyses to our γ nodes within species-specific networks (Figure 27, Attachment 14 and Attachment 15).

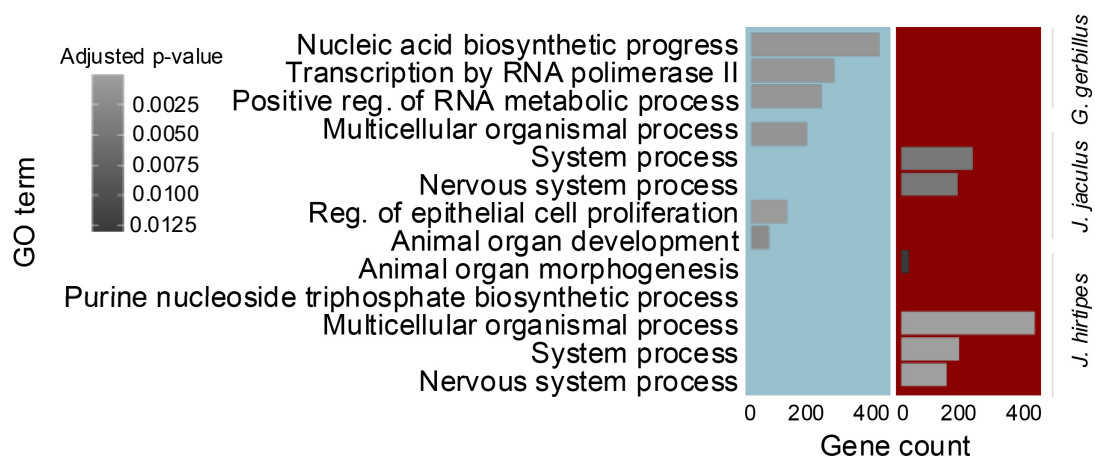


Figure 27 - Top three terms for Gene Ontology enrichment results in metabolism (no enriched terms) and cognition (grey) associated gamma nodes across species and tissues. Hippocampus in blue and Liver in dark red.

For the metabolic liver networks, we found no significant GO or KEGG enrichment in any species (Figure 27), suggesting that rewiring genes are not consistently associated with metabolic regulation in our species networks. In contrast, cognitive hippocampal networks revealed a gradient in GO enrichment: *G. gerbillus* showed 22 GO terms (0 KEGG), *J. jaculus* had 8 GO terms (0 KEGG) and *J. hirtipes* had none, mirroring the heterothermy level of the species.

This pattern aligns with the heterothermy gradient: *G. gerbillus*, the species with highest heterothermy, showed the strongest enrichment, *J. jaculus* showed a modest profile and *J. hirtipes* showed none. The GO terms in *G. gerbillus* were strongly associated with transcriptional regulation and chromatin remodelling, including gene expression regulation, chromatin dynamics and RNA biosynthesis. These enrichments suggest transcriptional plasticity, which may support cognitive flexibility under high heterothermy, although not directly indicative of cognitive function without neuronal context. In *J. jaculus*, enriched terms reflected broader developmental and stimulus response programs, including morphogenesis and regulation of organismal processes. This profile suggests engagement in general developmental and adaptive responses, which may intersect with neuronal function but lack specificity for cognition. Taken together, these results suggest that hippocampal γ node rewiring is functionally linked to transcriptional plasticity in *G. gerbillus*, potentially supporting cognitive adaptability under high heterothermy. In contrast, *J. jaculus* exhibited broader developmental enrichment, while *J. hirtipes* lacked functional enrichment entirely, reflecting a possible heterothermy-associated gradient in hippocampal transcriptional adaptability.

To address Q5, we examined whether the genes responsible for network rewiring are functionally enriched in pathways related to metabolism and cognition. Our findings provide partial support for this hypothesis. Specifically, rewiring genes showed significant enrichment in transcriptional processes within the hippocampus, suggesting a strong association with cognitive regulation. However, no comparable enrichment was observed for metabolic regulation, indicating that the contribution of rewiring genes may be functionally biased toward neural rather than metabolic plasticity. These results (Q5), highlight a domain-specific role of gene network flexibility in response to thermoregulatory variation.

5.4. Discussion

Heterothermy levels and thermal downregulation

Our findings confirm marked interspecific differences in daily body temperature fluctuation, with *G. gerbillus* exhibiting the highest downregulation (mean $\Delta T = -7.19^{\circ}\text{C}$) and *J. hirtipes* the lowest (-0.72°C). These results are consistent with prior studies indicating that mammals inhabiting environments with low productivity and high thermal variability benefit from adopting heterothermic strategies (Bastos *et al.*, 2021; Geiser, 2008). The association between environmental unpredictability and daily torpor has been previously demonstrated in free-ranging rodents (Boratyński *et al.*, 2018), reinforcing the notion that heterothermy can function as a buffer against environmental stress (Geiser

& Brigham, 2012; Nowack *et al.*, 2020). Moreover, the observed sex-specific differences in temperature regulation, with females consistently showing greater downregulation suggest a potential role for hormonal modulation in thermogenic responses (Refinetti, 2020), a pattern that warrants further exploration considering sex-biased torpor sensitivity reported in small mammals (Levesque *et al.*, 2016).

Transcriptomic divergence and tissue-specific trade-offs

Differential gene expression analysis revealed tissue- and function-specific gene expression across species between night and day. The liver, a key site for metabolic regulation, exhibited extensive expression plasticity in *G. gerbillus* and *J. hirtipes*, albeit through contrasting mechanisms. While *G. gerbillus* appears to preserve homeostatic expression across thermogenic pathways despite large temperature variation, *J. hirtipes* shows disproportionately high expression changes despite minimal body temperature variation, likely reflecting alternative physiological triggers such as feeding or circadian digestion rhythms (Heldmaier *et al.*, 2004). In contrast, hippocampal expression profiles of cognition-related genes showed convergent stability across species, even though having subtle variations. *J. jaculus* exhibited the highest proportion of differentially expressed cognition-associated genes, suggesting that even under low heterothermic regimes, cognitive modulation may be a priority. This aligns with evidence that hippocampal function, including memory consolidation and plasticity, is modulated by circadian and thermoregulatory cues (Ruby *et al.*, 2008; Gerstner & Yin, 2010).

Network connectivity and functional compartmentalisation

The architecture of co-expression networks revealed a dissociation between network size and connectivity, with cognitive networks consistently larger and more connected than metabolic ones, yet this significance can be explained due to the gene datasets having different sizes (2375 and 1528 genes, respectively). Across tissues and conditions, we observed significant species-dependent differences in network connectivity, which is a proxy for regulatory flexibility. *G. gerbillus* exhibited higher overall connectivity within both metabolic and cognitive networks under day and night conditions, suggesting greater neurophysiological plasticity. In contrast, *J. hirtipes* displayed more rigid network architecture, with pronounced night-enhanced connectivity in metabolic pathways, indicative of a strategy focused on maintaining metabolic homeostasis. Additionally, while *G. gerbillus* maintained balanced connectivity between cognitive and metabolic modules, *J. hirtipes* showed a stronger emphasis on metabolic regulation, particularly during the n

Such patterns mirror findings in other torpor-adapted taxa, where flexibility in gene regulatory networks supports transitions between active and suppressed physiological states (Boyles *et al.*, 2011; Geiser, 2004). The circadian enhancement of hippocampal connectivity at night observed in *J. jaculus* and *G. gerbillus* aligns with nocturnal increases in glial activity and neurogenesis, previously reported in heterothermic species (Gerstner & Yin, 2010), further supporting a functional synchronisation between thermoregulation and brain plasticity.

Stability-flexibility trade-offs and the role of rewiring

Our CoDina analysis quantified a clear trade-off between network stability (α) and flexibility (γ), with *G. gerbillus* exhibiting a high γ -to- α ratio, consistent with predictions that greater heterothermy promotes transcriptional rewiring. The strongest differential co-expression response was observed in the liver, with marked shifts across heterothermic phases. This supports previous findings suggesting that peripheral organs, such as the liver, serve as early modulators of metabolic transitions during torpor-arousal cycles (Geiser & Turbill, 2009). The liver's high γ node proportion in *G. gerbillus* contrasts with its stability in *J. hirtipes*, again reflecting their divergent strategies: metabolic versatility under extreme fluctuations versus metabolic steadiness under mild ones. These findings extend earlier models of heterothermy evolution, suggesting that plastic co-expression networks may act as genomic facilitators of energy-saving states (Lovegrove, 2017; Bastos *et al.*, 2021).

Functional annotation and transcriptional plasticity in cognition

Notably, GO enrichment of γ nodes in hippocampal cognitive networks revealed a heterothermy-correlated gradient: *G. gerbillus* showed enrichment in chromatin remodelling and transcriptional regulation, *J. jaculus* showed broader developmental plasticity, and *J. hirtipes* showed no significant enrichment. These results align with theories that cognitive robustness under fluctuating internal states may depend on epigenetic mechanisms (Preußner *et al.*, 2017), with heterothermic species relying more heavily on dynamic transcriptional control to preserve neural function during torpor-arousal transitions. The absence of significant enrichment in liver metabolic γ nodes across all species suggests a decoupling between structural rewiring and classical metabolic pathways. Instead, core metabolic stability may be maintained via conserved topologies, with functional adjustments emerging through post-transcriptional mechanisms (Nespolo *et al.*, 2011) or via hormonal and enzymatic regulation rather than large-scale network reconfiguration.

Evolutionary implications

Our results reinforce the notion that daily heterothermy is a potent driver of transcriptional and network flexibility, with broader implications for understanding mammalian adaptation to climate unpredictability. The association between environmental temperature variability and heterothermy levels in mammals has been previously proposed as an evolutionary buffer against extinction (Geiser & Turbill, 2009; Bastos *et al.*, 2021). Our findings suggest this buffering may extend to molecular plasticity mechanisms, particularly in neural circuits governing behaviour and memory. Future comparative analyses across broader phylogenetic scales may help elucidate whether the flexibility-stability trade-off we observed represents a generalised rule in thermoregulation-linked transcriptomics, or whether it is confined to specific ecological or taxonomic groups.

5.5. Conclusion

This study provides integrative evidence that species with varying degrees of daily heterothermy adopt fundamentally distinct strategies of gene network organisation to navigate the energetic challenges of fluctuating environments. In line with our hypothesis, species exhibiting higher body temperature downregulation displayed more flexible gene co-expression networks, particularly in cognitive domains, reflecting a systems-level mechanism of adaptive plasticity. Across five analytical dimensions, we observed consistent associations between heterothermic capacity and molecular flexibility. First, thermoregulatory variation among species was mirrored by divergent transcriptional responses, with sex-specific thermal downregulation patterns suggesting fine-tuned physiological regulation. Second, transcriptomic divergence in metabolic and cognitive pathways revealed species-specific allocations of regulatory investment, reflecting distinct prioritisation strategies. Third, co-expression network architectures varied in connectivity across tissues and circadian phases, with cognitive networks consistently more connected at night and metabolic networks exhibiting greater interspecific plasticity. Fourth, a clear trade-off between network stability and flexibility emerged, with higher level heterothermic species favouring dynamic, rewired networks, while more homeothermic species maintained rigid architectures. Finally, functional enrichment of rewiring genes in the hippocampus, but not in liver networks, underscored the domain-specific nature of molecular adaptability, pointing to cognitive regulation as a primary locus of thermal plasticity. Overall, these findings highlight a fundamental evolutionary tension between stability and flexibility in physiological regulation. Species with greater heterothermy appear to offset energetic constraints through dynamic transcriptional and network-level plasticity, whereas those with limited thermal fluctuations rely on more robust but potentially less adaptive regulatory frameworks. By demonstrating that gene co-expression network architecture reflects thermoregulatory strategies, our study offers new insights into the molecular underpinnings of potentially adaptive resilience in mammals, with implications for predicting species responses to climatic variability.

Chapter 6. Ontogenic onset of endothermy in laboratory mouse pups in a controlled environment

6.1. Introduction

The ontogenetic transition from poikilothermy to endothermy represents a fundamental physiological milestone in the developmental shift in mammals and birds. In mammals, this transition has traditionally been assumed to occur around weaning, a developmental point associated with physiological and behavioural independence (Koteja 2000; McNab 2002; Koteja 2004). However, evidence for this shift occurring at weaning remains limited, across species (Koteja 1991; Hayes J.P. and Garland, Jr. 1995). This raises a key question: is endothermy established abruptly at weaning, or does it emerge gradually through an interplay between developmental and environmental factors? Recent research suggested that this poikilothermy-endothermy transition may reflect a multifactorial developmental trajectory, rather than a single-stage shift (Garland and Carter 1994; Angilletta and Sears 2003; Geiser 2021).

Endothermy, defined as thermoregulation by a controlled high rate of heat production (Bligh and Johnson 1973) enables animals to remain active across variable thermal environments (Polymeropoulos *et al.* 2018). The emergence of endothermic physiology is often linked to extended parental care, which supports fast offspring growth and survival, but imposes intense energy maternal demands during pregnancy and lactation (Koteja 2000; Farmer 2003; Kemp 2005; Tattersall *et al.* 2016). Evolutionary and ecological pressures may have shaped divergent development trajectories of thermoregulation, with endothermy offering biochemical and physiological advantages under thermally variable conditions (Polymeropoulos *et al.* 2018). The transition from neonatal poikilothermy, in which body temperature varies with ambient conditions, to active adult endothermy marks a key functional shift in thermal autonomy (Blumberg, 1997). In altricial mammals, such as rodents, who are not capable of homeothermic endothermic regulation at birth and for their first days of life, endothermy is shaped by metabolic maturation, neuroendocrine signalling, and behavioural adaptations (Zhao *et al.*, 2004; Okuda & Koizumi, 1989).

In newborn altricial mammals, such as *Mus musculus*, the acquisition of homeothermy is thought to coincide with weaning, which is typically around the third postnatal week, when maternal nursing ceases (around 3rd week of age in mice; Lagerspetz, 1966; Nagy, 1993). During the first postnatal weeks, mouse pups produce insufficient internal heat and depend on maternal contact, warm milk ingestion, huddling and ambient external warmth. Although brown adipose tissue (BAT) is present from birth, its thermogenic function is immature and cannot maintain stable body temperature without external input (Lagerspetz, 1966). The onset of metabolic thermogenesis, by increased basal metabolism, shivering, and BAT activity, depends on maturation of sympathetic and endocrine control mechanisms (Nagy, 1993, Cannon & Nedergaard, 2004). For instance,

effective BAT-mediated thermogenesis requires sympathetic activation and thyroid hormone signalling, which only mature postnatally (Lagerspetz, 1966; Nagy, 1993).

Behavioural thermoregulation plays a central role in mitigating heat loss prior to the establishment of full homeothermic control in developing mammals. Neonates huddle and seek warmth from their environment, while older pups progressively rely on internal (metabolic) thermogenesis and fur insulation (Nagy, 1993). Given that neonates heavily rely on maternal warmth, maternal body temperature likely modulates early thermal states and heat retention capacity in pups. However, the precise timing and mechanistic underpinnings of this developmental transition remain unresolved across taxa (Gropp, 1972; Mortola & Frappell, 2000; Blumberg, 1997; Zhao *et al.*, 2004). Our aim is to uncover how temperature regulation develops under standardised conditions, informing our understanding of developmental thermogenesis. To accomplish this, we monitored the development of genetically defined, fully homozygous, inbred laboratory mice (*Mus musculus*, C57BL/6J), from birth to young adulthood, under controlled laboratory conditions (room temperature, diet, nesting material).

Hypothesis:

Endothermic homeothermic regulation sets around weaning age (postnatal day 21), developing gradually, rather than abruptly, from neonate-typical poikilothermy.

Research questions:

- Q1 - Does endothermy emerge as a gradual developmental process or through an abrupt physiological shift?
- Q2 - Do male and female pups exhibit divergent thermogenic trajectories, consistent with sex-specific developmental regulation?
- Q3 - Does maternal body temperature influence the initial thermal state and heat loss dynamics of the pups?
- Q4 - Does nest presence modulate thermoregulatory performance, and if so when do pups become independent from it?

6.2. Materials and methods

Animal husbandry and experimental design

This study was preregistered at the 'Animal Study Registry' on October 2nd, 2023 (DOI: 10.17590/asr.0000328) and carried out afterwards. Eight breeding pairs of C57BL/6J mice (descendants from Colony 16, Jackson Laboratory) were used to produce 11 litters, yielding 82 live pups (mean litter size: $\bar{x}=7.45$). The litter was treated as the experimental unit (N=11). While this sample size would only allow detecting considerably large, standardized effect sizes, our pilot study suggested such large effects were to be expected, particularly large temperature variations as a result of the treatment applied (separation from mother and placement in an open arena, causing either hypo- or (stress- and activity-induced) hyperthermia, depending on whether pups expressed, respectively, a poiki- or endotherm physiology). Moreover, pooling data from an average

of 7–8 pups per litter (each pup being an observational units) increased the precision of our estimates. Due to the workload this experiment was divided into 3 experimental batches (cycles).

Breeding, housing, and postnatal care

Breeding pairs were housed in type II cages (Tecniplast) with corncob bedding (changed weekly), Teklad Harlan 2014S pellets (Envigo, UK), nesting material (shredded paper and huts), and water bottles filled with sterilized water. Environmental conditions were maintained at 21–22°C ($\pm 1^\circ\text{C}$) with 45–65% relative humidity and a 12-hour light/dark cycle (~ 100 lux during the light phase). Males were removed following pregnancy detection, based on vaginal plug observation within three days post-mating. Pregnant females were isolated in individual cages during late gestation. After parturition, pups remained with their mothers until postnatal day 21 (standard weaning time at our facility). At weaning, pups were split by sex and housed under identical environmental conditions with added enrichment (e.g., cardboard tubes). Perinatal and daily health monitoring assessed key indicators such as respiratory movements, umbilical cord integrity, and milk spot visibility.

Thermal monitoring and assessment

From birth to postnatal day 35, pups were assessed twice daily (once during the light period, once during the dark period). Each pup was placed in an arena (Drönjös, 33x37x33cm) lined with a cotton absorbent pad (to avoid direct contact with a smooth, cold surface). Superficial temperature (TS) was recorded using a Thermal Expert TE-EV1 infrared camera (IR resolution: 640 × 480 pixels). Initial recordings spanned 3 minutes and were extended to 6 minutes after postnatal day 12. From postnatal day 10, subcutaneous body temperature (Tsc) was recorded using thermosensitive passive integrated transponder (PIT) tags (1.5 × 10.7 mm, ThermoChip Mini) subcutaneously implanted under the scruff under 1.5–2.0% isoflurane anaesthesia (in oxygen) via 14G needles (5-min procedure). An RFID scanner (GPR Plus Handheld, Biomark) enabled contactless subcutaneous temperature tracking and minimized handling stress. Three thermal images were acquired at each timepoint (0, 3, and 6 minutes; TS0, TS3 and TS6) and averaged at each timepoint. If fewer than three images were available ($\sim 1.5\%$ of cases), the average was calculated from the available frames. Thermal images (8481 images) were processed using ThermoLabAnimal software (v3.4; (Franco *et al.* 2019), which automatically removes the thermal background, highlights each animal's thermograms, and provides mean and median temperature values. Between minutes 3 and 6, pups were gently separated to remove thermal stabilization from huddling. PIT tags reading provided Tsc at the start (Tsci) and end (Tscf) of each thermal imaging session. Metadata integration incorporated litter ID, experimental cycle, light phase, timestamp, room and rack temperatures, along with observational notes.

Nest removal experiment

From postnatal day 18 to day 34, nest removal experiments were conducted every two days following thermal assessments, exclusively during the mice's active phase (the dark light phase). Pups were randomly assigned to either housing with or without nesting material, with randomization across sex applied after day 21. Both groups had corncob bedding, with animals given nesting material were provided with shredded paper and a cardboard hut). Pups were randomly placed for 30 minutes before weaning (P21) or 60 minutes thereafter in either cage. Temperature readings (T_s and T_{sc} were taken at both the start (minute 0, $T_{sc0nest}$, TS_{0nest}) and end (minute 30 or 60, $T_{sc30/60nest}$, $TS_{30/60nest}$) of each nest restriction session. At each time point, three thermal images were recorded per pup and averaged. If fewer than three images were available (~1.7% of cases), the average was calculated from the available images.

Data analysis

To test our hypotheses on the development of endothermy and its physiological modulators, we used non-linear spline mixed-effects models applied to longitudinal temperature data. Superficial (T_s) and subcutaneous (T_{sc}) body temperature were monitored from birth to postnatal day 35. Primary predictors of body temperatures tested included postnatal day (P_{day}), ambient room temperature (TA , $\Delta \pm 1^\circ C$), and light phase (mice_light phase; light/dark), with additional covariates such as litter size, initial and final temperature (T_{sci}/T_{si} and T_{sc_f}/T_{sf}), and their differences (T_{sc_diff}/T_{s_diff}), litter_ID was included as random factor (testing its intercept), with random intercepts and, where relevant, random slopes (e.g., for ambient temperature, TA). Correlations between random intercepts and slopes were extracted to evaluate individual variation in the effect of TA on ΔT_{sc} . Sex of the pups and the dam's subcutaneous body temperature (T_{scdam}) were introduced to test for sex-specific thermogenic trajectories (Q2) and maternal effects (Q3), respectively. Nest presence effects (Q4) were analysed from postnatal day 18 onward by comparing nest (C) and no nest (E) groups using P_{day} as a continuous predictor ($df=3$) and group as fixed effect. To evaluate whether thermal stability emerged gradually or abruptly (Q1), we modelled inflection points in T_{sc} and TS using spline derivatives and assessed slope changes before and after these points. All models were fitted using Restricted Maximum Likelihood (REML) and accounted for repeated measures using litter_ID as a random effect. Visualisation and data wrangling were performed using the R packages "ggplot2" and "dplyr". Animals with congenital defects or health issues were excluded. Statistical analyses were conducted in R (v4.3.1) using "lme4", "splines", "lmerTest", and "emmeans" across all hypotheses (Q1–Q5).

Ethical considerations

All procedures adhered to the EU Directive 2010/63/EU for the protection of animals used for scientific purposes and following Portuguese legislation, were approved by the i3S' Animal Welfare Body. Only FELASA and DGAV authorized personnel handled and carried out scientific procedures to the animals (28/22_09_2022).

6.3. Results

Endothermic capacity emerges before weaning in laboratory mice

To evaluate whether thermoregulatory independence emerges before weaning (P21, Figure 28), we quantified superficial (TS) and subcutaneous body temperature (Tsc) changes in mouse pups raised under standardised laboratory conditions across the first 35 postnatal days.

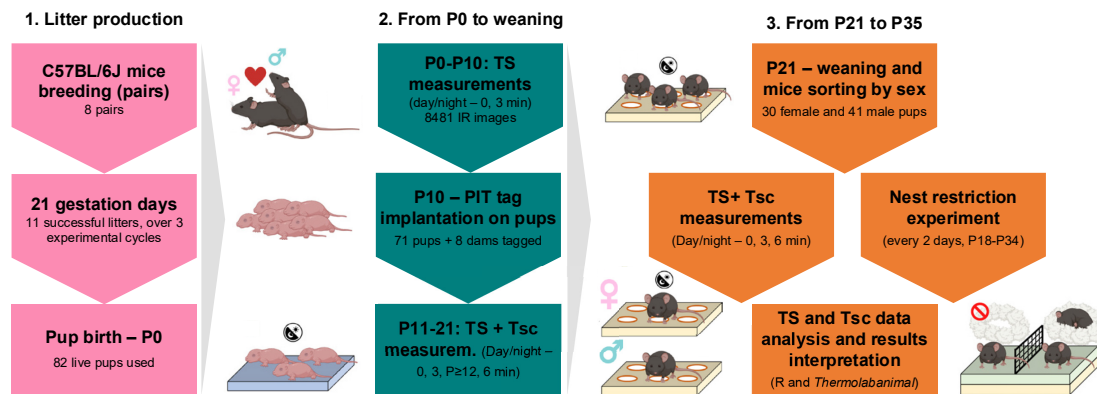


Figure 28 - Methodology overview. Flowchart summarizing the methodology processes and analyses conducted in this paper for the assessment of the shift between the poikilothermy to endothermy in laboratory mice.

No significant differences in thermal patterns were observed across the three experimental cycles (Figure 29). And the cycle was introduced in the analyses as random factor (blocks).

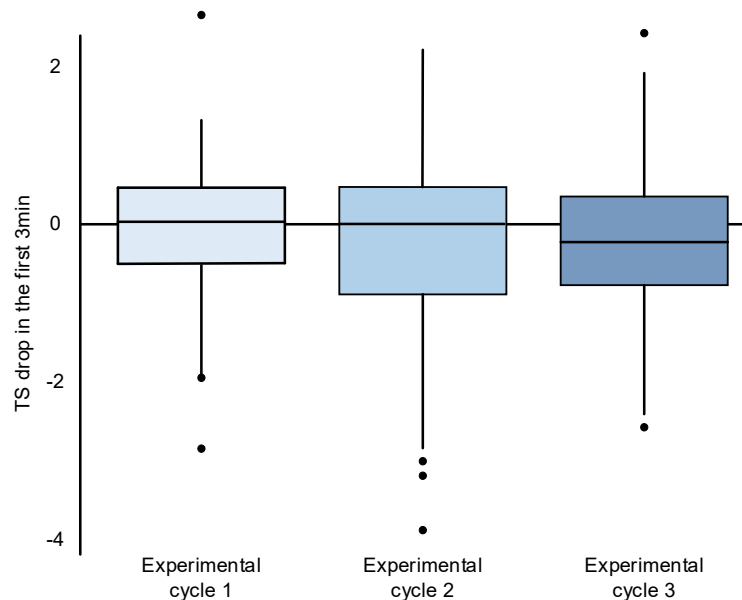


Figure 29 - Variation of superficial temperature (TS) drop among the three experimental cycles.

Analysis of TS changes during short-term isolation (0–3 min) revealed that pups shifted from net heat loss to net heat gain between postnatal day 14 and 15 (postnatal day, $P = 14.842$; Figure 30) suggesting this to be the onset of endogenous thermogenesis and the emergence of active thermoregulatory responses. This transition likely reflects a developmental threshold where behavioural arousal (Blenkuš, U., *et al.* 2022) and stress-induced thermogenic mechanisms (e.g., sympathetic activation and increased locomotor activity) become functionally integrated (Oka *et al.* 2001; Vianna and Carrive 2005).

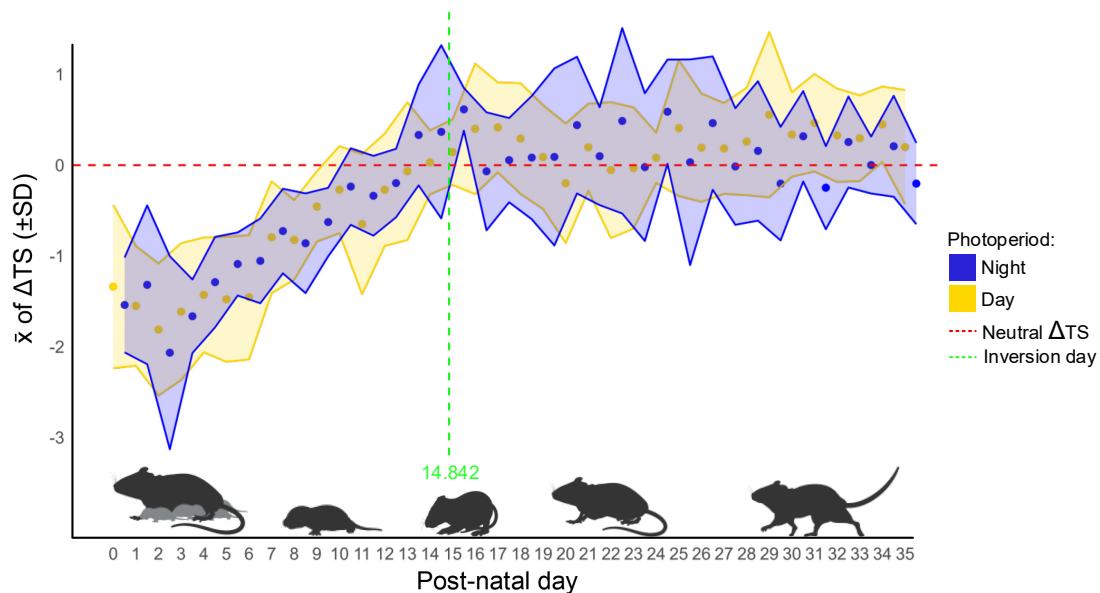


Figure 30 - Mean superficial (TS) temperature variations in laboratory mice for night and day periods across 35 days. Variation of TS mean across 0-35 postnatal day and light phase in the first 3 minutes, with transition point to endothermic thermal control at postnatal day, $P \approx 14.842$ (± 0.744).

At P14, mean ΔTS_{3-0} was minimal (day: $0.03^{\circ}\text{C} \pm 0.35^{\circ}\text{C}$; night: $0.36^{\circ}\text{C} \pm 0.95^{\circ}\text{C}$), indicating thermoregulatory equilibrium (day TS_{140} : 30.58°C ; TS_{143} : 30.61°C ; night TS_{140} : 30.187°C ; TS_{143} : 30.55°C). TS values during the dark period exceeded diurnal ones until P18, after which this trend reversed, suggesting a developmental transition in photoperiodic thermoregulation. The model, based on (651 observations for our 11 litters, showed a good overall fit (REML=1287.5, scaled residuals: -3.47 to 3.84). Within this well-fitting framework, developmental day (P_{day}) emerged as a highly significant non-linear predictor of surface temperature ($p < 0.001$), underscoring its pivotal role in the ontogeny of thermoregulation. Within the same model ambient temperature (TA) positively predicted TS loss ($\beta = 0.153$, $p = 0.0069$), likely reflecting indirect thermal feedback via the arena environment. Mice light phase (light/dark) ($\beta = -0.011$, $p = 0.833$) and litter size ($\beta = -0.020$, $p = 0.534$) had no significant effect. A strong negative correlation ($r = -0.975$) was observed between the intercept (baseline TS when all predictors are at reference level) and ambient temperature, reflecting the dominant influence of ambient temperature on the baseline thermal state of the pups. Together, these results identify postnatal development and ambient temperature as key determinants of surface thermoregulation, while light phase and litter size appear

negligible. Mean Tsc differences (initial–final) indicate that pups shift from heat loss to gain at P13–14 (mean postnatal day, 13.94, i.e. ~P14; Figure 31, Attachment 16).

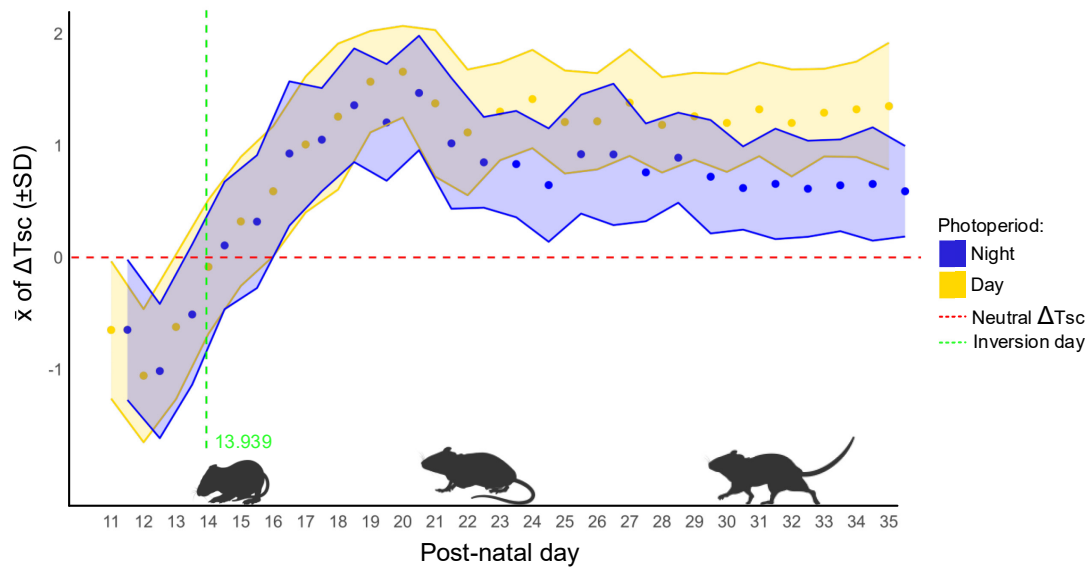


Figure 31 - Mean subcutaneous body (Tsc) temperature variations and standard deviation in laboratory mice for night and day periods across 24 days. Variation of Tsc mean across 11-35 post-natal day and light phase in the first 3 minutes, with transition point to endothermic thermal control at postnatal day, $P \approx 13.938$.

At P14, ΔT_{sc} (subcutaneous) was $-0.084^{\circ}\text{C} \pm 0.60^{\circ}\text{C}$ (day) and $0.11^{\circ}\text{C} \pm 0.57^{\circ}\text{C}$ (night), again suggesting thermal neutrality (day $T_{sc14i} = 36.08^{\circ}\text{C}$, $T_{sc14f} = 35.99^{\circ}\text{C}$; night $T_{sc14i} = 36.18^{\circ}\text{C}$, $T_{sc14f} = 36.29^{\circ}\text{C}$). Tsc values were consistently lower at night than during the day across development. Mean Tsc differences (initial–final) indicate that pups shift from net heat loss to stable temperature or temperature rise around ~P14 (~13.9 days of age; Fig. 4). The model to account for ΔT_{sc} , yielded 2695 observations. (REML=4648.8, scaled residuals: -5.35 to 5.46). Postnatal day was a highly significant nonlinear predictor of ΔT_{sc} ($p < 0.001$), highlighting the critical role of development in thermoregulation. Light phase significantly modulated Tsc loss ($\beta = -0.257$, $p < 0.001$), with greater stability during daytime. Neither litter size ($\beta = -0.007$, $p = 0.803$) nor ambient temperature ($\beta = -0.032$, $p = 0.248$) significantly contributed to ΔT_{sc} . A high negative correlation ($r = -0.929$) between intercept and the slope of and TA again highlighted the strong baseline effect of ambient temperature. In sum, ΔT_{sc} reflects developmental maturation and photoperiodic modulation, while litter size and ambient temperature appear to have negligible predictive value in this context.

Overall, when taken together, TS and Tsc analyses point to the shift to thermoregulatory competence in laboratory mice occurring prior to P15, earlier than hypothesised.

Endothermy in laboratory mice develops progressively, but not uniformly

To determine whether endothermy develops gradually or abruptly in laboratory mice, we analysed the trajectories of superficial (ΔTS) and subcutaneous body (ΔT_{sc}) temperature differences across postnatal development. Following identification of inflection points in their respective curves (Figure 30 and Figure 31), we compared the slopes of the mean developmental trends before and after these points. For ΔTS , the inflection point occurred at postnatal day, $P_{day} \approx 14.8$ ($SE = 0.744$), marking a transition from progressive heat loss to thermoregulatory stabilisation, with a marked slope change: prior to the inflection, the slope was ≈ 0.139 ($SE = 0.137$) but dropped to ≈ 0.001 thereafter (Figure 30). This steep-to-flat pattern suggests an abrupt transition in surface temperature dynamics. In contrast, ΔT_{sc} exhibited a more continuous shift. The inflection point occurred earlier, at $P_{day} \approx 13.94$, with a shallower pre- and post-inflection slope (0.041 to 0.016), suggesting more stable internal temperature dynamics. This relative smoothness likely reflects effective thermoregulatory buffering, as internal body temperature is less sensitive to ambient fluctuations than surface temperature (Figure 31).

These findings suggest that while surface temperature changes are abrupt (likely as the result of peripheral vasoconstriction can be a thermoregulatory mechanism in and of itself), the maturation of core thermoregulation in laboratory mice follows a predominantly gradual trajectory, consistent with progressive internal acquisition of endothermic capacity (Q1).

Thermoregulatory development does not differ between sexes

To evaluate whether thermoregulatory development differs between male and female pups, we extended the ΔT_{sc} REML model by including sex as a fixed effect (Figure 31). The model yielded 2698 observations ($REML=4644.9$, scaled residuals: -5.35 to 5.48) indicating a robust fit. Random intercept variance across litters was $\sigma^2 = 0.01088$ ($SD = 0.104$), and residual variance was $\sigma^2 = 0.32084$ ($SD = 0.566$), suggesting that variation was mostly attributable to fixed effects and residual error, rather than litter-level differences. Postnatal day (P_{day}) remained a strong nonlinear predictor of ΔT_{sc} ($ns(P_{day})$, $df = 3$; $p < 0.001$), confirming the developmental origin of thermal regulation. Light phase also retained significance ($\beta = -0.2617$, $p < 0.001$), with night-phase ΔT_{sc} being lower than day-phase values. In contrast, sex had no significant effect on ΔT_{sc} ($\beta = 0.00395$, $p = 0.870$), nor did litter size ($\beta = -0.00602$, $p = 0.827$) or ambient room temperature ($\beta = -0.03214$, $p = 0.253$). Correlations among fixed-effect predictors were generally low ($r < 0.3$), with no substantial collinearity detected between P_{day} , light phase, litter size, and sex. However, ambient temperature showed a strong negative correlation with the model intercept ($r = -0.930$), indicating its dominant influence on baseline ΔT_{sc} . Sex was not analysed for TS , as individual identification was not feasible via thermal imaging or visual markings. Postnatal maturation and light phase, rather than sex, are the main determinants of ΔT_{sc} during this period (Q2)

Maternal body temperature influences thermal state and stability in pups

To assess whether early pup thermoregulation was influenced by maternal temperature at the time of pup removal from the nest, we modified the main REML model to analyse the influence of maternal body temperature (Tscdami, Figure 32). We applied this updated model to four outcomes: initial pup body temperature (Tsci, Figure 32 – A), initial surface temperature (TS0, Figure 32 – C), and their respective differences after short-term exposure (ΔT_{sc} and ΔTS , Figure 32 – B and D), using data from pups prior to weaning (P0–P21). The models were fit to 1278 observations for Tsc and 1071 for TS, yielding REML criteria of 2185.9 (Tsci), 2423.9 (TS0), 1856.4 (ΔT_{sc}), and 1971.6 (ΔTS). Scaled residuals ranged between -5.78 and 5.40 across models, indicating good model fit. Random effects analyses showed low between-litter variance in all models, with most variation attributable to within-litter residuals. Postnatal day was a significant non-linear predictor in all models ($p < 0.001$ for Tsci, TS0, and ΔT_{sc} ; $p < 0.005$ for ΔTS), confirming the strong developmental progression of thermoregulatory capacity.

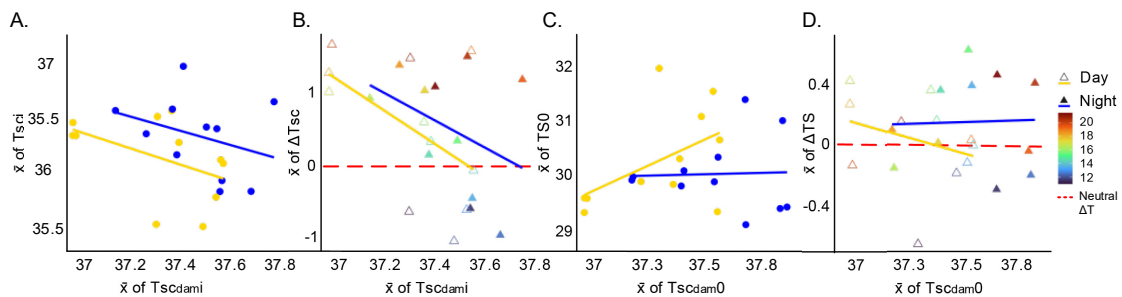


Figure 32 - Influence of mean initial dam body temperature (Tscdami) on, A. mean pup initial body temperature ($\bar{x}T_{sci}$); B. mean pup body temperature variation ($\bar{x}\Delta T_{sc}$); C. mean pup initial (min 0) superficial temperature ($\bar{x}TS_0$) and D. mean pup superficial temperature variation ($\bar{x}\Delta TS$). Yellow colour and empty triangles portrait day values, blue and full triangles, night values and the rainbow colour range indicates postnatal day associated.

Initial subcutaneous maternal temperature (Tscdami) significantly predicted initial subcutaneous pup temperature (Tsci; $\beta = 0.0757$, $p = 0.0189$) and the proportional change in pup temperature over time (ΔT_{sc} ; $\beta = -0.1376$, $p < 0.001$). Here, ΔT_{sc} refers to the relative change in Tsci between the beginning and end of the measurement period, normalised to the initial value. This indicates that higher maternal temperatures are associated with higher initial pup temperatures and reduced relative heat loss over time. Initial surface temperature (TS0) showed a marginal trend towards significance ($\beta = 0.0861$, $p = 0.0594$), though the effect size was small, suggesting limited predictive value in the context of the model. In contrast, change in superficial temperature, ΔTS was not affected by initial subcutaneous maternal temperature ($\beta = 0.0181$, $p = 0.621$), suggesting that maternal influence primarily modulates internal rather than peripheral thermoregulation. These findings suggest that pups with warmer dams had higher initial internal and surface temperatures, and reduced internal heat loss, supporting a role for maternal thermal contact in stabilising core temperature. In contrast, surface heat dynamics were less strongly influenced, likely due to greater exposure to environmental conditions. Light phase had a significant effect on Tsci ($\beta = 0.1670$, $p < 0.001$), ΔTS ($\beta = 0.1142$, $p = 0.0051$), and TS0 ($\beta = -0.1477$, $p = 0.0034$), but not ΔT_{sc} . This pattern suggests increased internal temperatures and peripheral loss during the dark period,

alongside reduced surface baselines, highlighting photoperiodic modulation. Ambient laboratory room temperature had a significant positive effect only on ΔT_{sc} ($\beta = 0.1394$, $p = 0.0135$). Litter size negatively influenced T_{sci} ($\beta = -0.1460$, $p = 0.0189$) and positively influenced T_{SO} ($\beta = 0.2668$, $p = 0.0120$), suggesting that pups from larger litters may retain more surface heat while exhibiting lower subcutaneous temperatures. Pairwise correlations among the fixed-effect predictors (light phase, ambient temperature, litter_ID, T_{scdami}) were generally low, indicating that their contributions to the models are statistically independent. However, maternal temperature consistently showed a strong negative correlation with the model intercept ($r = -0.723$ to -0.758), consistent with its strong influence on baseline temperature values across models.

These results highlight a critical role for maternal body temperature in stabilising internal temperature and reducing core heat loss in developing pups. This maternal effect likely buffers immature thermoregulatory systems and supports the gradual acquisition of autonomous endothermy before weaning (Q3).

Nest removal does not impair thermoregulation after postnatal day 18

We did not find nest removal to impair thermoregulation after postnatal day 18. Because PIT-tag readings of T_{sc} measurements allowed us to track individuals within each litter, only T_{sc} temperature data were analysed at this stage (Figure 33).

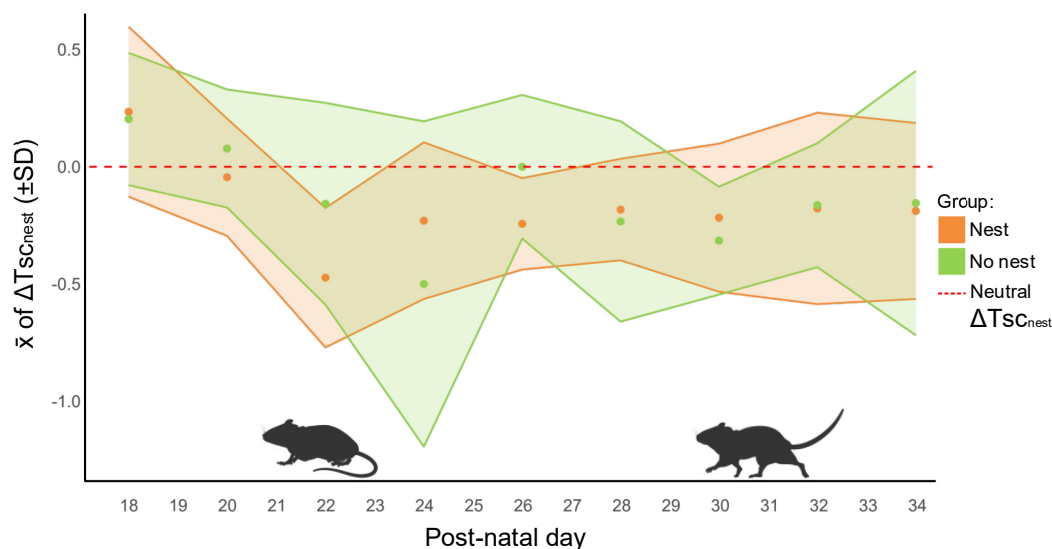


Figure 33 - Mean internal body nest (T_{scnest}) temperature variations and standard deviation in laboratory mice for night and day periods across 17 days. Variation of T_{sc} mean across 18-35 post-natal (every 2 days, 30min <P21; 60min >P21).

Superficial temperature (T_S) was excluded in this case, as thermal imaging did not allow reliable sex or identity tracking of individuals. Given the robustness of the PIT tag measurements and their direct link to core thermoregulation, ΔT_{sc} was deemed sufficient for addressing nest-related thermo-competence. To assess whether the presence of nesting material influences thermoregulation in older pups, we analysed T_{sc} variation

(ΔT_{scnest}) in response to nest removal every two days between postnatal day 18 and 34. In each litter, pups were divided into two groups and placed in separate compartments of a cage for 30 or 60 minutes: the control group received standard nesting material and a hut, while the experimental group was left without nesting material. Both compartments contained identical corncob bedding. This protocol was carried out exclusively during the dark phase to align with the pups' active period. We fit a REML model with group (nest vs. no nest), postnatal day (continuous predictor (df=3)), litter size and ambient room temperature (TA) as fixed effects, with litter_ID as a random effect (436 observations; Figure 33). The model yielded a REML criterion of 408.9, and scaled residuals ranged from -7.24 to 5.55, indicating acceptable model performance. Random intercept variance across litters was low ($\sigma^2 = 0.0065$, SD = 0.0806), with most variation attributed to residual and within-litter differences ($\sigma^2 = 0.1369$, SD = 0.3700). Postnatal day was a significant non-linear predictor of ΔT_{sc_nest} ($p = 0.0138$ and $p < 0.001$ for the first two spline components), though the third component was not significant ($p = 0.2602$), suggesting strong but uneven developmental modulation. Crucially, the presence or absence of nesting material had no significant effect on Tsc variation ($p = 0.4246$). Also, neither litter size ($p = 0.4648$) nor ambient temperature ($p = 0.3092$) was shown to have significant effects.

These findings indicate that nest presence does not significantly affect internal temperature loss after P18, supporting the interpretation that mice at this age are thermo-competent. Their ability to maintain stable body temperatures independent of nest structure aligns with the acquisition of endothermic autonomy in the third postnatal week (Q4).

6.4. Discussion

Contrary to what predicted in our primary hypothesis, the transition from poikilothermy to homeothermy does not occur around weaning (P21) but rather emerges through a gradual interplay of physiological and environmental factors, starting near P13 and stabilising by P15 (Q1). This aligns with prior studies in small mammals suggesting that thermogenesis, especially non-shivering thermogenesis via brown adipose tissue (BAT), matures gradually and is modulated by neuroendocrine development (Lagerspetz, 1966; Nagy, 1993; Cannon & Nedergaard, 2004). Superficial skin temperature (TS) appeared to shift more abruptly than subcutaneous temperature (Tsc), which may reflect peripheral vasoconstriction as a strategy to maintain internal body temperature stable, as well as direct contact with colder, room air, after loss of contact with the dam's body heat and removal from the stabilizing effect of huddling, both playing a key role in mitigating heat loss before internal thermogenesis becomes effective (Harshaw & Alberts, 2012; Glancy *et al.*, 2016). Importantly, our findings suggest that subcutaneous temperature offers a more reliable proxy for studying thermogenic maturation, although surface temperature monitoring by thermal imaging offers insight into thermoregulatory mechanisms (and is moreover the more ethically acceptable method for very early-age pups). The gradual shift in ΔT_{sc} , and its consistent modulation by postnatal age and light phase, but not by sex, litter size or ambient temperature, suggests a core endogenous mechanism underlying thermal autonomy. These data reinforce existing developmental models of

endothermic control that prioritise metabolic and endocrine maturation over simple environmental dependence (Blumberg, 1997; Zhao *et al.*, 2004). Although we predicted that males and females could diverge in their thermogenic profiles (Q2), particularly given prior reports of early sex differences in metabolism and hormonal activity, our data did not support this hypothesis. It is possible that sex-specific trajectories become more apparent later in development or under more ecologically variable conditions, as previously reported in wild rodents (Speakman & Krol, 2005). Nonetheless, our study suggests that the early developmental acquisition of endothermy follows a broadly conserved trajectory across sexes in C57BL/6 mice. Our third research question (Q3) was strongly supported: maternal body temperature at the time of removal from the home cage significantly predicted initial pup body temperature and internal thermal stability (T_{sci} and Δ T_{sc}). These findings confirm the role of maternal thermoregulation in buffering immature pups against ambient fluctuations and priming early homeostasis. While the influence on surface temperature (TS) was less robust, this is consistent with previous studies showing that TS (Harshaw & Alberts, 2012) is more susceptible to environmental exposure and posture than T_{sc}.

Absence of a significant interaction between day/night phase and Δ T_{sc} supports a steady, non-disruptive developmental pattern. Although the slope between Δ TS and the inversion point (when Δ TS reaches 0, changing from negative to positive) may appear abrupt, it is likely influenced by extrinsic factors. In early postnatal stages, pups remain immobile, huddled, and in constant contact with the mother and littermates, reducing exposure to ambient conditions and dampening superficial thermal fluctuations (Harshaw & Alberts, 2012). As pups develop motor ability and separate spatially within the arena, increased surface area exposure can exaggerate TS variation. This behavioural transition, alongside increased exploration and vertical positioning within huddles (“pup flow”; Glancy *et al.*, 2016; Alberts, 2007) can amplify surface fluctuations without reflecting underlying thermogenic capacity. In contrast, Δ T_{sc} provides a more direct measure of temperature regulation, partially insulated from surface-level confounders, like fur and fat. Its gradual change aligns with maturational processes underlying thermos-effector activation, including non-shivering thermogenesis, brown adipose tissue recruitment, and metabolic upregulation (Blumberg, 1997; Okuda & Koizumi, 1989; Lagerspetz, 1966). Moreover, previous work shows that core temperature control matures independently and often more slowly than surface regulation, reinforcing our interpretation that Δ T_{sc} reflects intrinsic thermophysiological development (Zhao *et al.*, 2004; Harshaw & Alberts, 2012).

Together, these findings refine our understanding of the ontogeny of endothermy in altricial mammals. Our results confirm that while behavioural and maternal inputs shape early thermoregulation, internal thermal autonomy develops progressively and occurs prior to weaning. This study provides a strong foundation for future research into the molecular and metabolic mechanisms that underlie this transition.

Limitations and future directions

While our thermal data reveal key developmental trends, some limitations must be acknowledged. We did not measure core body temperature, metabolic rate, BAT gene expression, or endocrine parameters (e.g., thyroid hormones, leptin), which are critical to fully characterising the maturation of thermogenesis and homeothermic endothermy. Inclusion of respirometry and transcriptomic profiling would allow mechanistic dissection of the pathways supporting ΔT_{sc} stability and energy homeostasis. Additionally, our use of a single inbred strain under controlled laboratory conditions limits ecological generalisability. On the other hand, the main goal of this study was to standardize intrinsic (genetic) and intrinsic (environment) factors as much as possible. Future studies should examine if similar thermogenic trajectories are found across multiple strains, as well as wild-derived mice, and whether, or to which extent, the nutritional status, stress, or environmental complexity modulate the acquisition of homeothermy. Moreover, due to welfare concerns and based on the assumption that endothermy would only arise around weaning, the effect of nest removal (Q4) was only measured after postnatal day 18. At this stage, pups were already thermo-competent, maintaining stable ΔT_{sc} values even in the absence of insulating nesting material. This approach, while ethically necessary, limited our ability to determine whether earlier nest deprivation would impact the onset of endothermic capacity. However, it is consistent with prior evidence indicating full BAT activation and metabolic insulation by the third postnatal week (Okuda & Koizumi, 1989). Despite these limitations, our study provides a reproducible experimental framework for tracking thermoregulatory development with fine temporal and individual resolution. These data contribute to a broader effort to integrate physiological, behavioural and genetic perspectives on endothermy evolution and development in mammals (Angilletta & Sears, 2003; Geiser, 2021; Garland & Carter, 1994).

6.5. Conclusion

Based on frequent measurements of subcutaneous (under the skin) and surface (above the skin) temperatures, our results point to the ontogenetic emergence of endothermy in mice being a gradual phenomenon which occurs before weaning, and modulated by both intrinsic and extrinsic factors, with body temperature (ΔT_{sc}) after a challenge (removal from nest, mother, and huddling with conspecifics) stabilising around postnatal day 14. The transition was smoother and more reliable in subcutaneous, and perhaps internal, body temperature than in superficial readings, which were more susceptible to environmental influence. No significant differences in ΔT_{sc} were observed between male and female pups, suggesting that thermal maturation follows a conserved trajectory during this developmental window. We moreover found maternal body temperature to significantly predict both the initial thermal state and heat loss dynamics of the pups, highlighting the buffering role of maternal warmth in early postnatal life and supports the idea that maternal physiology primes early homeostasis. These results provide a baseline developmental atlas for endothermy in altricial mammals and establish a platform for deeper metabolic and molecular investigation.

Chapter 7. General conclusions

7.1. Key findings

This thesis advances a comprehensive, integrative understanding of mammalian thermoregulation as a multi-level adaptive trait, shaped by environmental pressures and underpinned by molecular, physiological, developmental, and systems-level mechanisms. By combining comparative analyses across species, genomic and transcriptomic methodologies with controlled developmental experiments, the work collectively reinforces the view that heterothermy is not a marginal exception, but a widespread and evolutionary dynamic strategy enabling mammals to cope with energetic and thermal challenges of a changing planet. At the macroecological level (Chapter 3), we showed that environmental temperature and primary productivity are strong predictors of daily heterothermy expression. Mammals inhabiting colder, thermally variable, and resource-poor environments exhibited significantly lower minimum body temperatures. This indicates that energetic constraints and climatic unpredictability drive the evolution of energy-saving physiological states. Importantly, species adapted to arid climates did not exhibit the same degree of thermal flexibility, suggesting ecological limits to heterothermic adaptation and pointing to differential vulnerability to climate change. At the molecular evolutionary level (Chapter 4), our comparative genomic analysis revealed that thermoregulatory genes are broadly conserved across thermoregulatory strategies, but species with greater thermal flexibility exhibit distinct patterns of positive selection and network integration. Rather than single-protein modifications, we found evidence that a higher degree of protein interaction network complexity, particularly in hibernators and daily heterotherms, may provide the molecular foundation for the evolution of flexible thermoregulatory strategies. This suggests a shift from gene-centric models to network-centric frameworks when studying complex adaptive traits. At the systems and transcriptomic level (Chapter 5), we uncovered that species with greater body temperature fluctuations possess more flexible gene co-expression networks, particularly in brain regions associated with cognition. These species exhibited enhanced network rewiring, increased dynamic connectivity, and signatures of plasticity consistent with adaptive circadian and metabolic regulation. Conversely, more homeothermic species prioritized stability, indicating an evolutionary trade-off between flexibility and robustness at the regulatory network level. At the developmental level (Chapter 6), we demonstrated that endothermy emerges gradually, before weaning (postnatal day 14) in laboratory mice, stabilizing prior to the conventional expected weaning period for altricial mammals. Maternal body temperature significantly modulated pup thermal state and heat loss, but sex and nest presence did not. These results provide critical baseline data on the ontogeny of thermoregulation, reinforcing the role of maternal-pup thermal dynamics and highlighting developmental plasticity in establishing endothermic homeothermic competence.

7.2. Concluding remarks and prospects

Together, the findings presented in this thesis challenge the traditional dichotomy between homeothermy and heterothermy by illustrating a continuum of thermal regulation strategies that span across species, environments, and developmental stages. By integrating ecological, molecular, and developmental perspectives, this work reinforces the concept of heterothermy as a complex, plastic, and evolutionarily conserved strategy that enables survival under energetic constraints. Future research should prioritize expanding comparative frameworks to underrepresented taxa and climatic zones, particularly in tropical and arid regions where data is scarce. Integrating multi-omics approaches, including epigenomics and proteomics, could illuminate regulatory mechanisms underlying reversible phenotypic plasticity. Moreover, longitudinal studies of thermoregulatory development under experimental manipulations of ambient conditions will help delineate the role of early-life plasticity in shaping adult phenotypes. Finally, investigating cognitive and behavioral trade-offs associated with thermoregulatory flexibility will enhance our understanding of the evolutionary costs and benefits of heterothermy. These future directions are critical not only for advancing basic science, but also for predicting species responses to climate change, conservation prioritization and identifying molecular targets of adaptive resilience.

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Appendix

A. Attachments chapter 4

Attachment 1 – Information regarding number of orthologues per species and genes.

Gene/ N° of spp w/ orthologues	Gene common name	absent in:
ENSMUSG000 00001435 (11 spp.)	<i>Col18a1</i>	<i>C. lanigera</i> , <i>C. porcellus</i> , <i>D. ordii</i> , <i>J. jaculus</i> , <i>M. auratus</i> , <i>M. marmota marmota</i> , <i>N. galili</i> , <i>O. degus</i> , <i>P. maniculatus bairdii</i> , <i>S. vulgaris</i>
ENSMUSG000 00001729 (20 spp.)	<i>Akt1</i>	<i>D. ordii</i>
ENSMUSG000 00001773 (20 spp.)	<i>Folh1</i>	<i>U. parryii</i>
ENSMUSG000 00001930 (18 spp.)	<i>vwf</i>	<i>D. ordii</i> , <i>M. auratus</i> , <i>M. caroli</i>
ENSMUSG000 00004098 (12 spp.)	<i>Col5a3</i>	<i>C. lanigera</i> , <i>C. porcellus</i> , <i>H. glaber</i> , <i>I. tridecemlineatus</i> , <i>M. marmota marmota</i> , <i>N. galili</i> , <i>O. degus</i> , <i>S. vulgaris</i> , <i>U. parryii</i>
ENSMUSG000 00004951 (18 spp.)	<i>Hspb1</i>	<i>M. caroli</i> , <i>M. marmota marmota</i> , <i>M. ochrogaster</i>
ENSMUSG000 00005237 (19 spp.)	<i>Dnah2</i>	<i>M. marmota marmota</i> , <i>S. vulgaris</i>
ENSMUSG000 00005952 (18 spp.)	<i>Trpv1</i>	<i>M. auratus</i> , <i>N. galili</i> , <i>Rattus norvegicus</i>
ENSMUSG000 00014158 (20 spp.)	<i>Trpv4</i>	<i>H. glaber</i>
ENSMUSG000 00016356 (19 spp.)	<i>Col20a1</i>	<i>C. porcellus</i> , <i>D. ordii</i> , <i>M. spretus</i>
ENSMUSG000 00016529 (21 spp.)	<i>Il10</i>	NA
ENSMUSG000 00018507 (19 spp.)	<i>Trpv2</i>	<i>C. lanigera</i> , <i>M. pahari</i>
ENSMUSG000 00020063 (19 spp.)	<i>Sirt1</i>	<i>M. marmota marmota</i> , <i>P. maniculatus bairdii</i>
ENSMUSG000 00020660 (20 spp.)	<i>Pomc</i>	<i>M. marmota marmota</i>
ENSMUSG000 00020889 (20 spp.)	<i>Nr1d1</i>	<i>P. maniculatus bairdii</i>
ENSMUSG000 00020893 (17 spp.)	<i>Per1</i>	<i>D. ordii</i> , <i>J. jaculus</i> , <i>O. degus</i> , <i>P. maniculatus bairdii</i> ,

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ENSMUSG000 00021250 (20 spp.)	<i>Fos</i>	<i>D. ordii</i>
ENSMUSG000 00021756 (20 spp.)	<i>Il6st</i>	<i>P. maniculatus bairdii</i>
ENSMUSG000 00022383 (20 spp.)	<i>Ppara</i>	<i>O. degus</i>
ENSMUSG000 00023912 (18 spp.)	<i>Ucp4/Slc2 5a27</i>	<i>J. jaculus, M. auratus, M. pahari</i>
ENSMUSG000 00025064 (20 spp.)	<i>Col17a1</i>	<i>C. lanigera</i>
ENSMUSG000 00025630 (19 spp.)	<i>Hprt1</i>	<i>D. ordii, M. marmota marmota</i>
ENSMUSG000 00026072 (9 spp.)	<i>Il1r1</i>	<i>C. lanigera, C. porcellus, D. ordii, H. glaber, I. tridecemlineatus, J. jaculus, M. auratus, M. marmota marmota, N. galili, O. degus, S. vulgaris, U. parryii</i>
ENSMUSG000 00026820 (18 spp.)	<i>Ptges2</i>	<i>D. ordii, M. marmota marmota, N. galili</i>
ENSMUSG000 00029238 (18 spp.)	<i>Clock</i>	<i>J. jaculus, M. auratus, M. pahari</i>
ENSMUSG000 00029994 (20 spp.)	<i>Anxa4</i>	<i>M. caroli</i>
ENSMUSG000 00030827 (21 spp.)	<i>Fgf21</i>	NA
ENSMUSG000 00030987 (20 spp.)	<i>Stim1</i>	<i>J. jaculus</i>
ENSMUSG000 00031075 (20 spp.)	<i>Ano1</i>	<i>O. degus</i>
ENSMUSG000 00031105 (21 spp.)	<i>Ucp5/Slc 25a14</i>	NA
ENSMUSG000 00031710 (19 spp.)	<i>Ucp1</i>	<i>M. auratus, O. degus</i>
ENSMUSG000 00031762 (15 spp.)	<i>Mt2</i>	<i>C. lanigera, C. porcellus, D. ordii, H. glaber, M. spretus, O. degus, S. vulgaris</i>
ENSMUSG000 00031765 (13 spp.)	<i>Mt1</i>	<i>C. lanigera, D. ordii, H. glaber, I. tridecemlineatus, M. marmota marmota, O. degus, S. vulgaris, U. parryii</i>
ENSMUSG000 00032769 (21 spp.)	<i>Trpa1</i>	NA
ENSMUSG000 00032942 (21 spp.)	<i>Ucp3</i>	NA
ENSMUSG000 00033685 (21 spp.)	<i>Ucp2</i>	NA
ENSMUSG000 00036251 (21 spp.)	<i>Trpm8</i>	NA
ENSMUSG000 00038393 (20 spp.)	<i>Txnip</i>	<i>J. jaculus</i>
ENSMUSG000 00042678 (4 spp.)	<i>Myo15a</i>	<i>Cricetulus griseus, C. lanigera, C. porcellus, D. ordii, H. glaber, I. tridecemlineatus, J. jaculus, M. auratus, M. marmota marmota, M. ochrogaster, M. spicilegus, N. galili, O. degus, P. maniculatus bairdii, Rattus norvegicus, S. vulgaris, U. parryii</i>
ENSMUSG000 00043029 (21 spp.)	<i>Trpv3</i>	NA
ENSMUSG000 00046179 (21 spp.)	<i>E2f8</i>	NA
ENSMUSG000 00046949 (18 spp.)	<i>Nqo2</i>	<i>D. ordii, I. tridecemlineatus, N. galili,</i>

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ENSMUSG000 00052387 (21 spp.)	<i>Trpm3</i>	NA
ENSMUSG000 00055116 (21 spp.)	<i>Arntl</i>	NA
ENSMUSG000 00055866 (20 spp.)	<i>Per2</i>	<i>S. vulgaris</i>
ENSMUSG000 00056380 (10 spp.)	<i>Gpr50</i>	<i>C. lanigera</i> , <i>C. porcellus</i> , <i>D. ordii</i> , <i>H. glaber</i> , <i>I. tridecemlineatus</i> , <i>J. jaculus</i> , <i>M. marmota marmota</i> , <i>M. pahari</i> , <i>O. degus</i> , <i>S. vulgaris</i> , <i>U. parryii</i>
ENSMUSG000 00056749 (20 spp.)	<i>Nfil3</i>	<i>C. porcellus</i>
ENSMUSG000 00057722 (18 spp.)	<i>Lepr</i>	<i>C. lanigera</i> , <i>D. ordii</i> , <i>N. galili</i> ,
ENSMUSG000 00059201 (16 spp.)	<i>Lep</i>	<i>C. lanigera</i> , <i>C. porcellus</i> , <i>D. ordii</i> , <i>H. glaber</i> , <i>O. degus</i> ,
ENSMUSG000 00059970 (19 spp.)	<i>Hspa2</i>	<i>H. glaber</i> , <i>M. spicilegus</i>
ENSMUSG000 00063358 (21 spp.)	<i>Mapk1</i>	NA
ENSMUSG000 00068614 (20 spp.)	<i>Actc1</i>	<i>D. ordii</i>
ENSMUSG000 00072235 (16 spp.)	<i>Tuba1a</i>	<i>C. lanigera</i> , <i>I. tridecemlineatus</i> , <i>M. auratus</i> , <i>M. pahari</i> , <i>P. maniculatus bairdii</i> , <i>U. parryii</i> ,
ENSMUSG000 00029167 (21 spp.)	<i>Ppargc1a</i>	NA

Attachment 2- Gene name, number of species with orthologues and number of species with signs of positive selection according to their thermoregulation strategy (blue – hibernators, orange – daily heterotherms and green homeotherms)

Gene	N° spp. with orthologs	Species with genes with signs of positive selection
<i>Actc1</i>	20	none
<i>Akt1</i>	20	none
<i>Ano1</i>	20	none
<i>Anxa4</i>	20	<i>M. auratus</i> , <i>C. porcellus</i> , <i>M. ochrogaster</i>
<i>Arntl</i>	21	<i>O. degus</i>
<i>Clock</i>	18	<i>M. auratus</i> , <i>C. griseus</i> , <i>R. norvegicus</i>
<i>Col17a1</i>	20	<i>M. auratus</i> , <i>D. ordii</i> , <i>C. porcellus</i> , <i>O. degus</i>
<i>Col18a1</i>	11	<i>H. glaber</i> , <i>J. jaculus</i> , <i>Cricetulus griseus</i>
<i>Col20a1</i>	19	<i>H. glaber</i> , <i>M. musculus</i> , <i>M. caroli</i> , <i>O. Degus</i> , <i>S. vulgaris</i>
<i>Col5a3</i>	12	<i>N. galili</i>
<i>Dnah2</i>	19	<i>M. auratus</i> , <i>D. ordii</i> , <i>J. jaculus</i> , <i>C. porcellus</i> , <i>M. ochrogaster</i> , <i>M. spicilegus</i> , <i>O. degus</i> , <i>R. norvegicus</i> , <i>C. griseus</i>
<i>E2f8</i>	21	<i>N. galili</i> , <i>R. norvegicus</i>
<i>Fgf21</i>	21	none
<i>Folh1</i>	20	<i>M. marmota marmota</i> , <i>C. griseus</i> , <i>M. caroli</i> , <i>M. ochrogaster</i> , <i>O. Degus</i>
<i>Fos</i>	20	<i>M. ochrogaster</i>
<i>Gpr50</i>	10	<i>N. galili</i>
<i>Hprt1</i>	19	<i>C. lanigera</i>
<i>Hspa2</i>	19	none
<i>Hspb1</i>	18	<i>H. glaber</i> , <i>J. jaculus</i> , <i>M. ochrogaster</i>
<i>Il10</i>	21	none
<i>Il1r1</i>	9	<i>N. galili</i>
<i>Il6st</i>	20	<i>M. auratus</i> , <i>D. ordii</i> , <i>C. porcellus</i> , <i>M. ochrogaster</i> , <i>R. norvegicus</i>

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<i>Lep</i>	16	none
<i>Lepr</i>	18	<i>R. norvegicus</i>
<i>Mapk1</i>	21	none
<i>Mt1</i>	13	<i>C. porcellus</i>
<i>Mt2</i>	15	<i>P. maniculatus bairdii</i>
<i>Myo15a</i>	4	none
<i>Nfil3</i>	20	none
<i>Nqo2</i>	18	none
<i>Nr1d1</i>	20	none
<i>Per1</i>	17	<i>M. auratus</i>
<i>Per2</i>	20	<i>I. tridecemlineatus, M. marmota marmota, J. jaculus, C. lanigera, N. galili, O. degus</i>
<i>Pomc</i>	20	<i>M. auratus, D. ordii, P. maniculatus bairdii, M. pahari, N. galili</i>
<i>Ppara</i>	20	none
<i>Ppargc1a</i>	21	<i>D. ordii</i>
<i>Ptges2</i>	18	<i>J. jaculus, M. ochrogaster</i>
<i>Sirt1</i>	19	<i>M. auratus, U. parryii, H. glaber, J. jaculus, R. norvegicus, C. griseus, C. porcellus, M. spicilegus</i>
<i>Stim1</i>	20	<i>D. ordii, S. vulgaris</i>
<i>Trpa1</i>	21	<i>I. tridecemlineatus, M. auratus, C. porcellus, O. degus</i>
<i>Trpm3</i>	21	none
<i>Trpm8</i>	21	none
<i>Trpv1</i>	18	<i>O. degus</i>
<i>Trpv2</i>	19	<i>M. auratus, D. ordii, H. glaber, C. porcellus, N. galili, O. degus</i>
<i>Trpv3</i>	21	<i>M. marmota marmota, U. parryii, D. ordii, C. griseus</i>
<i>Trpv4</i>	20	none
<i>Tuba1a</i>	16	<i>M. spretus</i>
<i>Txnip</i>	20	none
<i>Ucp1</i>	19	<i>H. glaber, J. jaculus</i>
<i>Ucp2</i>	21	none
<i>Ucp3</i>	21	none
<i>Ucp4</i>	18	<i>P. maniculatus bairdii</i>
<i>Ucp5</i>	21	none
<i>Vwf</i>	18	<i>C. lanigera, S. vulgaris, O. degus, J. jaculus, H. glaber and I. tridecemlineatus</i>

Attachment 3 - Extended results of the positively selected amino acid sites both concerning 21 species and their respective thermoregulation group

Species	Gene name	Valid PSS	Validated PSS	P-value	Group
<i>H. glaber</i>	Col18a1	4	1171 P 0.734 1174 Y 0.665 1260 I 0.761 1285 R 0.907	0.001026	HET
<i>J. jaculus</i>	Col18a1	6	312 T 0.833 417 Q 0.593 418 A 0.768 453 D 0.554 608 R 0.541 752 E 0.551	5.48E-58	HET
<i>H. glaber</i>	Hspb1	5	25 A 0.647 29 L 0.613 58 P 0.556 101 A 0.623 166 S 0.992**	0.046796	HET
<i>J. jaculus</i>	Hspb1	2	66 G 0.657 208 A 0.566	8.46E-11	HET
<i>M. musculus</i>	Col20a1	1	568 S 0.562	0.015209	HET
<i>H. glaber</i>	Col20a1	1	392 Q 0.764	0.000604	HET
<i>D. ordii</i>	Trpv2	1	94 V 0.639	1.24E-10	HET
<i>H. glaber</i>	Trpv2	14	33 P 0.513 84 N 0.548 145 A 0.622 276 A 0.539 285 L 0.568 300 D 0.533 408 P 0.523 409 R 0.615 468 G 0.741 472 Y 0.637 507 R 0.539 509 V 0.526 597 P 0.764 634 E 0.604	4.06E-06	HET
<i>H. glaber</i>	Sirt1	6	264 W 0.691 265 Q 0.999** 569 E 0.707 613 S 0.504 747 D 0.545	9.7E-17	HET
<i>J. jaculus</i>	Sirt1	7	260 N 0.520 261 T 0.658 586 D 0.923 601 Q 0.508 615 V 0.647 635 S 0.980* 669 R 0.571	3.71E-23	HET
<i>D. ordii</i>	Pomc	3	9 S 0.765 47 I 0.845 188 P 0.615	0.000439	HET
<i>P. maniculatus bairdii</i>	Pomc	1	6 Y 0.974*	0.036992	HET
<i>D. ordii</i>	Il6st	4	153 G 0.553 181 E 0.998** 473 V 0.967* 515 A 0.583	4.41E-10	HET
<i>P. maniculatus bairdii</i>	Ucp4	3	139 V 0.786 162 R 0.695 235 S 0.803	9.7E-19	HET

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<i>D. ordii</i>	Col17a1	4	481 S 0.759 1039 T 0.578 1192 V 0.611 1297 S 0.618	0.034972	HET
<i>J. jaculus</i>	Ptges2	2	72 A 0.997** 98 A 0.985*	7.24E-11	HET
<i>D. ordii</i>	Ppargc1a	7	127 N 0.541 181 H 0.553 330 V 0.510 418 D 0.961* 536 Q 0.520 670 A 0.582 765 F 0.545	1.36E-07	HET
<i>D. ordii</i>	Stim1	1	40 S 0.912	0.022415	HET
<i>H. glaber</i>	Ucp1	5	60 V 0.514 148 H 0.660 265 G 0.686 266 P 0.993** 305 V 0.995**	0.024784	HET
<i>J. jaculus</i>	Ucp1	2	107 S 0.830 177 K 0.978*	0.010272	HET
<i>P. maniculatus</i>	Mt2	2	16 A 0.724 28 S 0.605	0.043223	HET
<i>D. ordii</i>	Trpv3	3	258 K 0.861 530 F 0.531 531 H 0.994**	1.46E-08	HET
<i>J. jaculus</i>	Per2	8	767 S 0.836 826 G 0.608 1196 L 0.510 1290 Q 0.960* 1298 E 0.725 1304 Q 0.990** 1345 E 0.964* 1373 E 0.566	0.001876	HET
<i>J. jaculus</i>	vwf	31	175 L 0.978* 225 R 0.663 513 T 0.617 627 V 0.687 630 L 0.982* 632 Y 0.556 747 A 0.719 803 V 0.508 978 N 0.544 1006 L 0.726 1077 V 0.968* 1204 Q 0.530 1230 Q 0.605 1379 I 0.593 1508 Q 0.504 1509 V 0.579 1529 K 0.858 1622 S 0.744 1760 G 0.506 2016 K 0.933 2071 G 0.609 2080 G 0.539 2100 Q 0.665 2173 S 0.663 2298 G 0.753 2462 T 0.830 2463 T 0.922 2605 Q 0.527 2645 T 0.923 2676 E 0.993** 2693 T 0.542	2.61E-26	HET
<i>H. glaber</i>	vwf	43	43 T 0.507 82 Y 0.550 247 M 0.526 248 W 0.549 306 M 0.695 362 G 0.584 462 D 0.506 547 K 0.504 567 A 0.539 722 Y 0.551 802 L 0.523 897 H 0.517 905 A 0.547 928 V 0.541 1054 P 0.614 1072 K 0.503 1170 N 0.602 1331 V 0.965* 1448 I 0.509 1485 R 0.515 1502 A 0.540 1590 G 0.529 1712 Q 0.623 1720 P 0.594 1798 Q 0.543 1833 V 0.580 1866 R 0.542 1883 N 0.550 1888 Q 0.626 1947 L 0.552 2003 K 0.504 2155 Q 0.543 2424 K 0.538 2461 T 0.522 2578 R 0.511 2639 M 0.576 2659 K 0.504 2776 Y 0.564 2783 K 0.542 2809 D 0.571 2811 Q 0.543 2828 P 0.514 2856 K 0.980*	2.99E-10	HET
<i>D. ordii</i>	Dnah2	27	205 T 0.878 212 D 0.820 213 A 0.503 268 V 0.763 276 E 0.964* 278 T 0.538 283 T 0.543 670 Q 0.883 697 G 0.993** 768 W 0.881 786 R 0.673 1077 V 0.698 1187 S 0.983* 1369 L 0.901 1431 M 0.502 2165 T 0.929 2337 R 0.668 2548 I 0.687 2878 V 0.692 3016 L 0.619 3155 A 0.987* 3370 A 0.521 3712 N 0.689 3716 K 0.861 4187 N 0.699 4274 S 0.890 4375 P 0.522	7E-16	HET
<i>J. jaculus</i>	Dnah2	11	780 G 0.714 801 G 0.711 839 T 0.989* 1124 Q 0.726 1145 R 0.753 1699 H 0.802 1711 I 0.768 1743 L 0.592 1892 G 0.761 3012 N 0.948 4107 S 0.701	1.59E-56	HET
<i>M. marmota</i>	Folh1	1	616 N 0.881	0.001642	HIB
<i>M. auratus</i>	Trpv2	7	34 P 0.691 37 E 0.991** 42 G 0.852 430 H 0.630 520 S 0.652 666 S 0.633 708 L 0.826	8.68E-05	HIB
<i>M. auratus</i>	Sirt1	4	425 A 0.594 598 L 0.991** 660 A 0.626 760 F 0.507	3.7E-09	HIB
<i>U. parryii</i>	Sirt1	2	603 T 0.627 752 P 0.965*	1.16E-30	HIB
<i>M. auratus</i>	Pomc	4	5 C 0.654 18 L 0.723 69 P 0.510 102 R 0.781	0.003283	HIB
<i>M. auratus</i>	Per1	10	173 K 0.827 575 P 0.997** 576 R 0.861 577 L 0.998** 606 A 0.651 853 P 0.647 875 P 0.851 1201 L 0.862 1284 A 0.635 1311 S 0.605	1.63E-25	HIB
<i>M. auratus</i>	Il6st	5	278 K 0.651 294 P 0.559 396 V 0.996** 494 T 0.693 910 E 0.686	2.86E-11	HIB
<i>M. auratus</i>	Col17a1	16	186 N 0.590 216 I 0.978* 362 A 0.781 386 I 0.643 388 S 0.716 577 V 0.659 685 I 0.862 688 E 0.974* 689 Q 0.893 691 P 0.750 740 M 0.573 932 S 0.755 1396 S 0.697 1445 S 0.775 1464 G 0.730 1613 E 0.640 207 P 0.937	4.43E-74	HIB
<i>M. auratus</i>	Clock	1		1.47E-46	HIB
<i>M. auratus</i>	Anxa4	5	31 E 0.953* 98 T 0.989* 181 D 1.000** 182 A 0.951* 183 L 0.958*	4.6E-16	HIB
<i>I. tridecemlineatus</i>	Trpa1	3	315 Q 0.635 747 L 0.626 850 I 0.953*	1.47E-72	HIB
<i>M. auratus</i>	Trpa1	4	397 N 0.650 480 I 0.637 810 R 0.570 945 A 0.537	0.000169	HIB
<i>M. marmota</i>	Trpv3	5	342 R 0.566 377 S 0.840 556 Y 0.855 576 R 0.829 653 N 0.826	2.06E-25	HIB
<i>U. parryii</i>	Trpv3	3	335 N 0.875 404 T 0.776 518 P 0.863	6.55E-10	HIB
<i>M. marmota</i>	Per2	13	178 S 0.592 273 V 0.500 623 S 0.599 631 K 0.572 633 V 0.562 658 N 0.519 912 S 0.577 923 T 0.514 964 G 0.578 966 Q 0.537 1001 P 0.508 1224 L 0.548 1271 F 0.558	2.37E-07	HIB
<i>I. tridecemlineatus</i>	Per2	9	303 I 0.628 343 K 0.774 346 E 0.776 370 V 0.502 573 H 0.792 575 S 0.693 581 E 0.776 1022 V 0.769 1128 G 0.786	2.71E-16	HIB
<i>I. tridecemlineatus</i>	vwf	17	119 D 0.734 379 V 0.948 510 I 0.735 1186 A 0.654 1285 D 0.710 1304 H 0.805 1359 D 0.783 1402 F 0.790 1511 H 0.612 1531 M 0.500 1559 E 0.733 1791 Q 0.579 1821 I 0.901 1844 V 0.843 1878 P 0.766 2210 V 0.774 2376 V 0.759	3.01E-25	HIB
<i>M. auratus</i>	Dnah2	16	257 N 0.530 778 Y 0.541 823 Q 0.995** 1060 L 0.619 1075 L 0.540 1135 S 0.552 1403 D 0.606 1699 H 0.517 2809 I 0.507 3882 I 0.632 4268 A 0.602 4280 K 0.508 4307 I 0.502 4386 Y 0.603 4432 E 0.550 4510 D 0.617	7.32E-34	HIB
<i>C. griseus</i>	Col18a1	5	359 T 0.764 385 A 0.681 451 D 0.712 502 E 0.545 528 L 0.714	2.05E-30	HO

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<i>C. griseus</i>	Folh1	7	177 V 0.830 289 V 0.836 305 Q 0.802 343 K 0.757 410 K 0.802 619	1.34E-09	HO
			M 0.696 622 P 0.817		
<i>M. caroli</i>	Folh1	3	97 Q 0.769 566 V 0.741 573 F 0.767	0.001782	HO
<i>M. ochrogaster</i>	Folh1	3	135 E 0.534 328 P 0.563 572 T 0.588	0.003983	HO
<i>O. degus</i>	Folh1	3	322 K 0.828 338 N 0.853 657 D 0.821	0.000188	HO
<i>M. ochrogaster</i>	Hspb1	1	94 V 0.619	4.33E-07	HO
<i>O. degus</i>	Trpv1	1	277 W 0.875	0.0373	HO
<i>M. caroli</i>	Col20a1	6	341 Q 0.793 753 V 0.715 881 E 0.739 1026 A 0.653 1062 I 0.507 1161	1.30E-46	HO
			L 0.736		
<i>O. degus</i>	Col20a1	10	180 E 0.940 185 G 0.627 539 V 0.911 660 D 0.886 775 K 0.514 857	7.53E-25	HO
			D 0.935 947 G 0.969* 1419 L 0.561 1435 E 0.824 1436 P 0.950*		
<i>Sciurus vulgaris</i>	Col20a1	9	352 Q 0.846 409 I 0.514 586 S 0.540 675 E 0.535 740 A 0.556 814 L	1.79E-40	HO
			0.529 841 E 0.588 1085 L 0.568 1186 D 0.530		
<i>C. porcellus</i>	Trpv2	9	136 V 0.618 140 Q 0.557 143 V 0.537 152 Q 0.611 273 E 0.559 371 N	0.031231	HO
			0.627 467 L 0.668 505 V 0.568 559 L 0.629		
<i>N. galili</i>	Trpv2	5	7 P 0.525 139 L 0.515 395 L 0.519 713 T 0.971* 774 H 0.541	0.014762	HO
<i>O. degus</i>	Trpv2	11	2 T 0.536 147 I 0.506 263 A 0.691 284 S 0.513 342 P 0.621 357 V	2.94E-05	HO
			0.992** 411 F 0.653 430 H 0.506 536 F 0.673 577 R 0.580 677 W		
			0.678		
<i>M. ochrogaster</i>	Sirt1	2	58 R 0.855 63 G 0.905	0.017399	HO
<i>R. norvegicus</i>	Sirt1	10	8 D 0.998** 40 A 0.998** 42 A 0.958* 44 M 0.830 46 A 0.834 47 A	1.16E-35	HO
			0.888 602 A 0.751 615 S 0.876 778 R 0.730 787 P 0.903		
<i>Cricetus griseus</i>	Sirt1	2	286 S 0.981* 743 D 0.651	2.64E-08	HO
<i>C. porcellus</i>	Sirt1	3	534 P 0.798 590 S 0.560 625 S 0.774	6.39E-25	HO
<i>M. spicilegus</i>	Sirt1	2	646 N 0.933 734 G 0.831	4.65E-14	HO
<i>M. pahari</i>	Pomc	1	26 S 0.625	0.008461	HO
<i>N. galili</i>	Pomc	3	5 C 0.607 11 A 0.904 185 G 0.602	0.013913	HO
<i>M. ochrogaster</i>	Fos	3	176 D 0.516 277 F 0.582 280 S 0.961*	0.027199	HO
<i>C. porcellus</i>	Il6st	9	17 R 0.859 19 W 0.966* 22 Q 0.533 23 A 0.999** 24 L 0.525 329 E	6.05E-10	HO
			0.960* 343 P 0.911 344 P 0.658 401 L 0.563		
<i>M. ochrogaster</i>	Il6st	7	36 L 0.556 72 V 0.546 249 E 0.552 331 A 0.593 691 N 0.525 695 Q	0.011902	HO
			0.536 898 D 0.877		
<i>R. norvegicus</i>	Il6st	9	198 V 0.767 350 T 0.537 353 S 0.924 583 H 0.567 605 R 0.736	0.034918	HO
			611 D 0.558 625 P 0.593 700 G 0.613 833 N 0.510		
<i>C. porcellus</i>	Col17a1	10	417 D 0.884 749 Q 0.703 1036 D 0.957* 1194 Y 0.942 1270 S 0.979*	5.29E-21	HO
			1291 P 0.985* 1293 L 0.954* 1329 A 0.879 1497 N 0.788 1500 S 0.686		
<i>O. degus</i>	Col17a1	12	582 M 0.880 832 P 0.926 834 K 0.996** 835 P 0.982* 837 L 0.949	1.32E-32	HO
			864 R 0.696 987 P 0.561 1002 G 0.524 1008 V 0.879 1010 G 0.514		
			1206 S 0.892 1530 G 0.870		
<i>N. galili</i>	Il1r1	6	28 Y 0.627 181 K 0.864 441 E 0.553 560 V 0.729 569 A 0.850 571 T	0.039823	HO
			0.552		
<i>M. ochrogaster</i>	Ptges2	7	366 I 0.994** 368 P 0.683 369 W 0.997** 370 Y 0.995** 371 L 0.998**	3.6E-09	HO
			372 R 0.882 373 M 0.991**		
<i>C. griseus</i>	Clock	2	755 V 0.851 756 T 0.865	4.67E-05	HO
<i>R. norvegicus</i>	Clock	12	131 I 0.595 132 I 0.595 137 S 0.756 184 Y 0.625 320 N 0.583 517 F	0.013084	HO
			0.597 612 - 0.524 648 Q 0.622 679 Q 0.624 799 E 0.604 801 Q 0.624		
			802 L 0.612		
<i>C. porcellus</i>	Anxa4	4	58 N 0.565 143 Q 0.625 189 Q 0.625 326 V 0.938	0.026034	HO
<i>M. ochrogaster</i>	Anxa4	1	91 Q 0.564	6.92E-14	HO
<i>Sciurus vulgaris</i>	Stim1	2	301 K 0.505 744 L 0.942	0.021681	HO
<i>Sciurus vulgaris</i>	Ano1	5	178 H 0.835 372 N 0.517 464 K 0.916 546 S 0.533 1014 C 0.991**	3.73E-28	HO
<i>C. porcellus</i>	Mt1	6	10 G 0.919 17 S 0.702 22 K 0.987* 27 T 0.831 40 G 0.679 53 A	0.003147	HO
			0.911		
<i>C. porcellus</i>	Trpa1	11	55 F 0.547 186 A 0.771 298 I 0.611 306 D 0.546 308 V 0.576 350 S	0.01243	HO
			0.604 446 H 0.532 869 C 0.611 912 S 0.592 937 E 0.611 1010 K 0.588		
<i>O. degus</i>	Trpa1	8	154 K 0.553 200 S 0.579 216 A 0.661 245 K 0.503 479 D 0.989* 681 T	1.05E-20	HO
			0.576 960 F 0.557 1006 W 0.576		
<i>C. griseus</i>	Trpv3	4	40 P 1.000** 41 T 0.999** 42 K 1.000** 43 K 1.000**	9.48E-27	HO
<i>N. galili</i>	E2f8	8	5 K 0.540 137 P 0.515 497 S 0.627 657 F 0.506 678 S 0.614 697 I	1.75E-07	HO
			0.815 770 P 0.584 844 L 0.517		
<i>R. norvegicus</i>	E2f8	6	18 M 0.582 228 I 0.615 242 H 0.639 266 V 0.641 466 K 0.512 626 S	9.59E-06	HO
			0.649		
<i>O. degus</i>	Arntl	1	536 I 0.505	0.027086	HO
<i>C. lanigera</i>	Per2	6	222 A 0.624 500 K 0.940 904 K 0.573 1237 I 0.584 1311 T 0.547 1365	1.47E-08	HO
			D 0.974*		
<i>N. galili</i>	Per2	1	1159 P 0.515	1.50E-05	HO

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<i>O. degus</i>	Per2	1	843 V 0.737	0.000373	HO
<i>N. galili</i>	Gpr50	2	413 K 0.589 591 C 0.910	0.005285	HO
<i>R. norvegicus</i>	Lepr	2	365 H 0.813 964 E 0.820	0.04448	HO
<i>M. spretus</i>	Tuba1a	18	115 P 0.882 131 H 0.865 135 L 0.895 147 A 0.811 166 R 0.677 186 G 0.887 234 T 0.797 257 R 0.768 262 I 0.909 290 A 0.900 337 A 0.842 351 R 0.886 357 A 0.918 362 Y 0.879 363 R 0.895 420 M 0.913 446 A 0.842 468 M 0.879	5.64E-07	HO
<i>C. lanigera</i>	vwf	37	73 M 0.586 141 S 0.591 166 I 0.599 326 G 0.979* 328 E 0.564 441 L 0.582 510 I 0.544 616 T 0.570 618 S 0.524 668 I 0.575 753 H 0.600 756 M 0.896 901 A 0.975* 933 Y 0.530 1086 I 0.584 1145 H 0.561 1678 I 0.524 1686 F 0.589 1711 L 0.608 1803 P 0.550 1804 S 0.560 1960 R 0.564 1992 R 0.585 2077 S 0.617 2140 V 0.575 2177 Q 0.588 2180 L 0.590 2327 K 0.527 2367 D 0.546 2368 L 0.560 2489 D 0.591 2506 V 0.537 2510 Q 0.606 2597 V 0.535 2638 V 0.568 2640 V 0.589 2806 D 0.546	0.009287	HO
<i>S. vulgaris</i>	vwf	5	232 P 0.958* 1462 R 0.750 1661 V 0.508 1670 A 0.511 2459 G 0.505	0.037737	HO
<i>O. degus</i>	vwf	58	130 Q 0.560 153 Y 0.525 171 N 0.505 250 Q 0.563 334 S 0.555 383 K 0.542 394 D 0.572 403 S 0.511 499 V 0.524 514 V 0.510 557 G 0.570 589 D 0.555 618 S 0.501 719 P 0.520 726 R 0.522 733 A 0.562 738 Y 0.525 749 I 0.524 853 E 0.611 1038 I 0.505 1064 S 0.505 1090 T 0.584 1198 P 0.585 1212 H 0.513 1277 G 0.586 1382 Q 0.619 1517 G 0.506 1636 A 0.527 1803 P 0.538 1830 K 0.540 1853 S 0.580 1865 D 0.571 1923 K 0.520 1996 T 0.530 2001 N 0.506 2030 S 0.564 2039 K 0.540 2124 I 0.512 2128 N 0.515 2132 D 0.501 2190 V 0.524 2247 N 0.539 2327 K 0.500 2424 K 0.516 2449 Y 0.575 2466 L 0.514 2476 T 0.600 2479 P 0.571 2516 S 0.505 2536 P 0.601 2594 Q 0.607 2601 P 0.576 2607 N 0.562 2669 A 0.511 2678 K 0.562 2685 G 0.605 2705 K 0.586 2768 K 0.586	8.47E-15	HO

Attachment 4- Result of PSS analysis within the three thermoregulation strategies forebranch groups

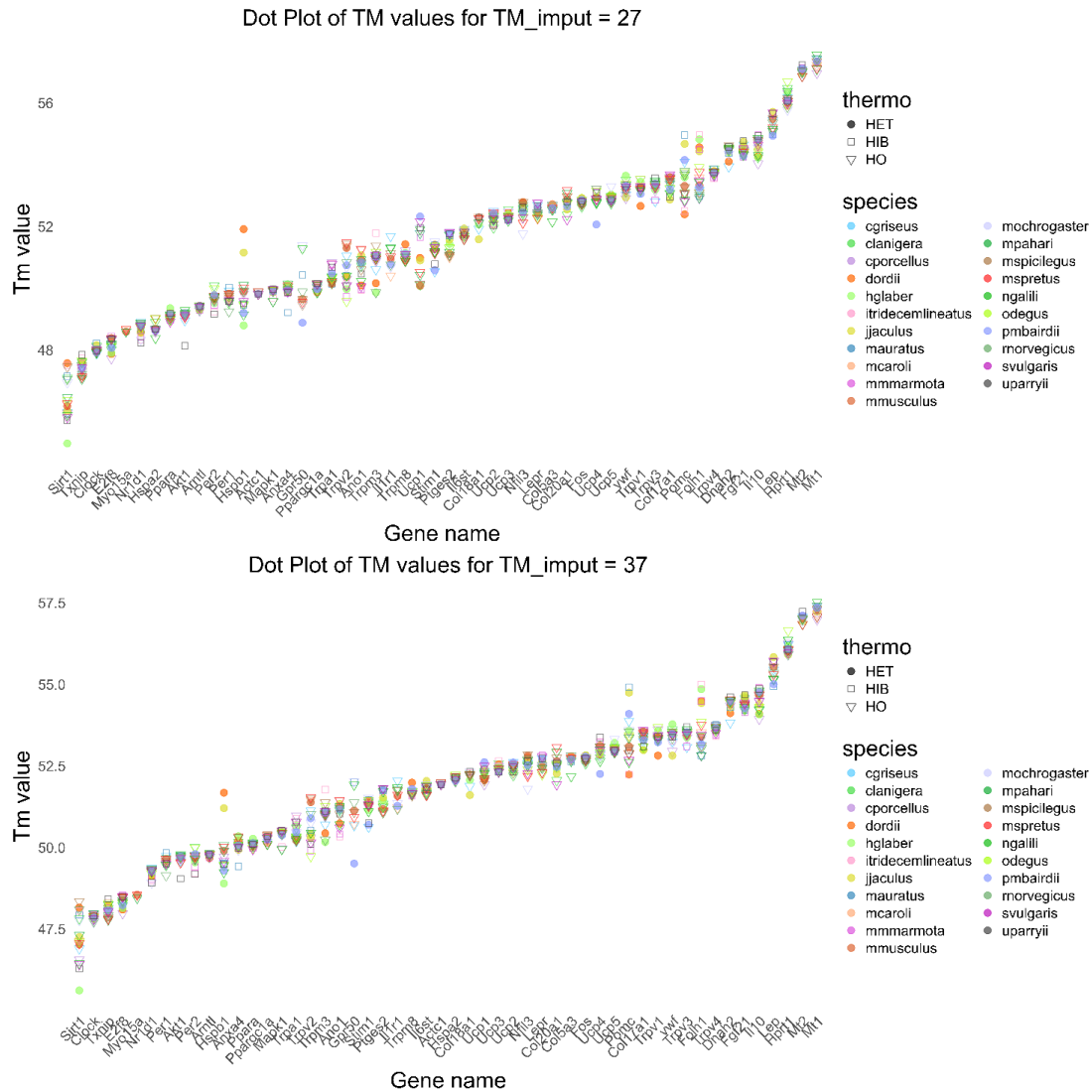
Analysis	Gene Ensembl ID	Gene name	Validate d N° PSS sites	Validated PSS	P value	Total PSS	%PSS (PSS/n° Spp)
Hibernators group (4 spp.)	ENSMUSG0000000193 0	vwf	30	119 D 0.545 156 S 0.569 212 D 0.692 361 E 0.568 379 V 0.847 510 I 0.564 595 S 0.580 733 A 0.688 897 H 0.556 905 A 0.575 1083 H 0.668 1268 L 0.673 1285 D 0.558 1304 H 0.672 1359 D 0.625 1402 F 0.628 1527 K 0.555 1559 E 0.562 1821 I 0.781 1834 I 0.842 1844 V 0.720 1878 P 0.635 1910 G 0.690 1943 N 0.515 1950 S 0.529 2210 V 0.625 2302 V 0.562 2349 A 0.574 2376 V 0.610 2482 Q 0.646 220 D 0.517 823 Q 0.969*	4.80E-11	92	23
	ENSMUSG0000000523 7	Dnah2	4	1115 S 0.654X 2587 P 0.550	1.76E-17		
	ENSMUSG0000002006 3	Sirt1	4	425 A 0.594 598 L 0.991** 660 A 0.626 760 F 0.507	1.19E-05		
	ENSMUSG0000002066 0	Pomc	4	5 C 0.654 18 L 0.723 69 P 0.510 102 R 0.781 173 K 0.827 575 P 0.997**	3.39E-02		
	ENSMUSG0000002089 3	Per1	10	576 R 0.861 577 L 0.998** 606 A 0.651 853 P 0.647 875 P 0.851 1201 L 0.862 1284 A 0.635 1311 S 0.605	1.32E-13		
	ENSMUSG0000002175 6	Il6st	5	278 K 0.651 294 P 0.559 396 V 0.996** 494 T 0.693 910 E 0.686	7.74E-07		
	ENSMUSG0000002506 4	Col17a1	16	186 N 0.590 216 I 0.978* 362 A 0.781 386 I 0.643 388 S 0.716 577 V 0.659 685 I 0.862 688 E 0.974* 689 Q 0.893 691 P 0.750 740 M 0.573 932 S 0.755 1396 S 0.697 1445 S 0.775 1464 G 0.730 1613 E 0.640	1.35E-33		
	ENSMUSG0000002923 8	Clock	1	207 P 0.937	6.22E-27		
	ENSMUSG0000002999 4	Anxa4	5	31 E 0.953* 98 T 0.989* 181 D 1.000** 182 A 0.951* 183 L 0.958*	8.31E-05		

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Heterotherms group (5 spp.)	ENSMUSG0000003276 9	Trpa1	3	315 Q 0.635 747 L 0.626 850 I 0.953*	1.03E-22	20	4
	ENSMUSG0000004302 9	Trpv3	5	342 R 0.566 377 S 0.840 556 Y 0.855 576 R 0.829 653 N 0.826	1.57E-11		
	ENSMUSG0000005586 6	Per2	4	892 L 0.799 971 V 0.638 1008 F 0.514 1077 Q 0.812	0.0192731		
	ENSMUSG0000006335 8	Mapk1	1	40 C 0.887	7.27E-07		
	ENSMUSG0000000143 5	Col18a1	6	312 T 0.833 417 Q 0.593 418 A 0.768 453 D 0.554 608 R 0.541 752 E 0.551	4.69E-07		
	ENSMUSG0000002916 7	Ppargc1a	7	127 N 0.541 181 H 0.553 330 V 0.510 418 D 0.961* 536 Q 0.520 670 A 0.582 765 F 0.545	2.62E-04		
	ENSMUSG0000002391 2	Ucp4	3	139 V 0.786 162 R 0.695 235 S 0.803	8.26E-03		
	ENSMUSG0000002006 3	Sirt1	4	542 H 0.823 602 E 0.886 635 S 0.742 742 G 0.681	3.81E-05		
	ENSMUSG0000002006 3	Sirt1	2	58 R 0.855 63 G 0.905	3.63E-07		
	ENSMUSG0000000143 5	Col18a1	5	359 T 0.764 385 A 0.681 451 D 0.712 502 E 0.545 528 L 0.714	2.17E-05		
Homeotherms group (12 spp.)	ENSMUSG0000000409 8	Col5a3	63	4 C 0.797 14 L 0.521 21 L 0.531 25 S 0.800 48 V 0.535 54 F 0.823 67 R 0.815 69 G 0.769 90 N 0.505 102 A 0.585 117 R 0.587 118 Q 0.604 119 L 0.551 122 A 0.562 128 G 0.600 136 P 0.563 137 L 0.553 150 R 0.523 151 V 0.571 169 Q 0.508 176 G 0.842 182 T 0.547 183 A 0.849 190 T 0.515 202 I 0.538 215 F 0.568 224 G 0.543 229 D 0.891 293 S 0.891 316 T 0.516 341 T 0.513 343 P 0.589 360 G 0.527 364 R 0.632 372 T 0.646 376 I 0.541 394 Q 0.512 428 D 0.543 431 L 0.563 545 R 0.622 635 S 0.807 659 N 0.514 734 N 0.568 737 L 0.564 770 P 0.576 833 K 0.529 1212 P 0.855 1222 P 0.549 1245 I 0.832 1263 A 0.541 1449 I 0.832 1452 P 0.829 1570 R 0.660 1587 R 0.786 1625 Y 0.521 1668 V 0.601 1672 T 0.551 1686 T 0.562 1713 N 0.503 1721 Q 0.504 1724 A 0.593 1726 T 0.633 1762 A 0.573	0.0005202	70	5.833333333

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Attachment 5 - Melting temperature (T_m) of thermoregulation genes across the 21 studied species, within the three thermoregulation groups, at both 27°C and 37°C. Species are identified with several different colours, where a dot, represents a daily heterotherm, an empty square, a hibernator and an empty triangle, a homeotherm species.



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Attachment 6- Extensive information regarding the conformational and functional conservation analysis. Species are identified according to thermoregulatory strategy (orange for daily heterothermy, blue for hibernators and green for homeotherms)

SPP/Thermo regulation	Gene	PSS	Observations	Gene observations
<i>C. griseus</i>	Col18a1	5	All PSS are concentrated on half of the protein, in both posterior and anterior faces. All are situated in regions of insufficient data or extremely variable regions. All five PSS are placed in loops.	The three protein structures share some horizontal conformation shape, yet vary on the vertical, due to different "spike-shaped" domains. All are low in α -helices and β -strands, being mostly constituted by loops. As for the in α -helix concentration the <i>H. glaber</i> is the one that shows the highest, followed by <i>J. jaculus</i> and <i>C. griseus</i> . All 3 structures present what seems to be a more dispersed base, increasing to a more globular shape with the described increase in α -helices.
<i>H. glaber</i>	Col18a1	4	2 groups of 2 PSS together in different positions of the protein, being one of the groups more peripheric. In one group, 1 PSS is in a α -helix and the other in a near loop and in the other 1 PSS is in a β -strand and the other in a near loop. As for conservation: 2 PSS are in a highly conserved place and other in regions of insufficient data.	
<i>J. jaculus</i>	Col18a1	6	All PSS are concentrated on half of the protein, in both posterior and anterior faces in near positions. All six PSS are placed in loops and in regions of insufficient data regarding to conservation.	
<i>C. griseus</i>	Folh1	7	6 of the 7 PSS are dispersed but concentrated in an upper part of the protein (considering a frontal view), being only 1 of them in the bottom part. In general, 2 of them are placed in loops, 4 in α -helices and 1 in a β -turn. All PSS are placed in regions of insufficient data regarding to conservation.	The 5 protein structures share conformation and are formed by a main globular structure, that is mostly constituted by α -helices and β -strands and a sequence almost resembling a "tail" formed by loops, in all cases. This "tail" assumes different conformations and shapes in all the 5 structures and only presents α -helices in <i>M. ochrogaster</i> .
<i>M. caroli</i>	Folh1	3	All the 3 PSS are in α -helices situated in a near site to each other. All PSS are placed in regions of insufficient data regarding to conservation.	
<i>M. marmota</i>	Folh1	1	The PSS is in a loop, in a variable region.	
<i>M. ochrogaster</i>	Folh1	3	The 3 PSS are in loops, being 2 in the upper of the protein (considering a frontal view), and the other more to a medium-bottom position. All are placed in regions of insufficient data.	
<i>O. degus</i>	Folh1	3	The 3 PSS are in loops. All PSS are placed in regions of insufficient data regarding to conservation.	
<i>H. glaber</i>	Hspb1	5	The 5 PSS are mostly concentrated in two regions of the protein, being 3 of them in a more peripheral part, with 2 in loops and 1 in a α -helix; and the other in a more medial upper part of the protein (in a frontal view) these are in a β -strand and in a loop, respectively. All PSS are placed in regions of insufficient data regarding to conservation.	All the 3 protein structures share a "V" shape conformation that is mostly formed by β -strands in a medial plane and loops with some α -helices (on the right side of the protein in a frontal view) in a more lateral plane.
<i>J. jaculus</i>	Hspb1	2	The 2 PSS are in opposite peripheric parts of the protein, both in loops. One is present in a variable region and the other in a region of insufficient data regarding to conservation.	
<i>M. ochrogaster</i>	Hspb1	1	The only PSS is in a posterior site (considering a frontal view) in a loop region that has insufficient data regarding to conservation.	

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<i>O. degus</i>	Trpv1	1	The only PSS is in a lateral site (considering a frontal view) in a loop region that has insufficient data regarding to conservation.	The protein structure has a "X" shape conformation, having an "antenna/arm" that is formed by α -helices and loops, as it is on the rest of its main X structure, except for the lower part that has a set of β -strands.
<i>M. musculus</i>	Col20a1	1	The only PSS in in a loop that has insufficient data regarding to conservation.	
<i>H. glaber</i>	Col20a1	1	The only PSS in in a loop that has insufficient data regarding to conservation.	All the structures are formed by small and scattered α -helixes, many loops and several distinct groups of β -sheets. Three of the five protein structures share conformation and cluster well (<i>M. musculus</i> , <i>H. glaber</i> and <i>M. caroli</i>), whereas the ones from <i>O. degus</i> and <i>S. vulgaris</i> have a rather different conformation and don't cluster well.
<i>M. caroli</i>	Col20a1	6	The 6 PSS are scattered in loops around the protein structure, being 2 in a proximate position in the upper right side and 3 in the bottom right side, respectively. All have insufficient data regarding to conservation.	
<i>O. degus</i>	Col20a1	10	The 10 PSS are scattered throughout the structure. 9 PSS are located in loops, whereas 1 PSS is in a β -sheet. 3 have variable conservation and 7 have insufficient data regarding to conservation	
<i>S. vulgaris</i>	Col20a1	9	The 9 PSS are scattered throughout the structure. 6 PSS are located in loops, whereas 3 PSS is in a β -sheets/strands. 1 PSS is in a conserved location and 8 PSS have insufficient data regarding to conservation.	
<i>C. porcellus</i>	Trpv2	9	The 9 PSS are scattered around the whole structure, mostly in areas of α -helixes (7) and loops (2). All of them reside in regions that have insufficient data regarding to conservation. Clusters with <i>H. glaber</i> .	
<i>D. ordii</i>	Trpv2	1	The only PSS is in an upper left lateral side of the protein (in a frontal view) in a α -helix region that has insufficient data regarding to conservation. The structure doesn't overlap with any cluster.	All the 6 protein structures have a "X" shape conformation, formed by α -helices and loops. The species that shows more PSS (<i>H. glaber</i>) is the only one lacking one "X leg" part in the protein, and it is a heterothermic species. Nonetheless, this gene shows positive selection in all thermoregulation groups.
<i>H. glaber</i>	Trpv2	14	This structure is missing one the "X" legs. All the 14 PSS are scattered in the structure, being 6 present in loops and the other 8 in α -helices. All are in regions that have insufficient data regarding to conservation. Clusters with <i>C. porcellus</i> .	
<i>M. auratus</i>	Trpv2	7	5 PSS are in loops and only two are in α -helices. Only one of these is in a high conserved site, being the rest in in regions that have insufficient data regarding to conservation. Clusters with <i>N. galili</i> and <i>O. degus</i> .	
<i>N. galili</i>	Trpv2	5	4 PSS are in loops, and one is in a α -helix, being all in regions that have insufficient data regarding to conservation. Clusters with <i>M. auratus</i> and <i>O. degus</i> .	
<i>O. degus</i>	Trpv2	11	The PSS are scattered around the whole structure, being 5 in loops and 6 in α -helices, being 1 in a variable site and the other 10 in sites that have insufficient data regarding to conservation. <i>M. auratus</i> and <i>N. galili</i> .	
<i>H. glaber</i>	Sirt1	6	3 PSS are present in α -helix and the other 3 in loops. 2 of the ones from α -helices are together. All the PSS are all in regions that have insufficient data regarding to conservation. Doesn't cluster.	This protein assumes several shapes: 1 - a more globular shape (for <i>J. jaculus</i> , <i>M. auratus</i> and <i>U. parryii</i>), 2 - a similar main globular shape with appendix
<i>J. jaculus</i>	Sirt1	7	All the PSS are in loops and are all in regions that have insufficient data regarding to conservation.	

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<i>C. griseus</i>	Sirt1	2	1 PSS in in a loop, that has insufficient data regarding to conservation and the other is in a α -helix, that is in a highly conserved region.	"arms" (for <i>C. porcellus</i> , <i>H. glaber</i> , <i>M. ochrogaster</i> and <i>R. norvegicus</i>) and 3- a miscellaneous wider form (<i>C. griseus</i> and <i>M. spicilegus</i>). Here we see that a heterothermic species (<i>R. norvegicus</i>) showed the higher number of PSS.
<i>C. porcellus</i>	Sirt1	3	All PSS are in loops, 1 in a conserved site and, the other in a variable site and the last has insufficient data regarding to conservation	
<i>M. spicilegus</i>	Sirt1	2	Both PSS are in loops, one in a variable site and the other has insufficient data regarding to conservation	
<i>M. auratus</i>	Sirt1	4	3 PSS are found in loops and 1 in a α -helix. All of them seem to be at the protein's surface. All have insufficient data regarding to conservation. Clusters with <i>J. jaculus</i> and <i>U. parryii</i>	
<i>M. ochrogaster</i>	Sirt1	2	Both PSS are in the protein's "arm" domain in loop sites. One is variable and other has insufficient data regarding to conservation. Clusters with <i>M. spicilegus</i> and <i>R. norvegicus</i> .	
<i>R. norvegicus</i>	Sirt1	10	6 PSS are present in loops and 4 in α -helices. 6 of the 10 PSS are concentrated in the upper region of the protein (frontal view). The rest assume surface positions in a protein's lateral "arm" (2 PSS) and in the bottom part of the protein (2 PSS). 1 PSS is in a conserved site and 9 PSS have insufficient data regarding to conservation. <i>M. ochrogaster</i> and <i>M. spicilegus</i> .	
<i>U. parryii</i>	Sirt1	2	Both PSS are present in loops. 1 is in a variable site and the other has insufficient data regarding to conservation. Clusters with <i>M. auratus</i> and <i>J. jaculus</i> .	
<i>D. ordii</i>	Pomc	3	2 PSS are in α -helices and 1 in a loop. 1 PSS in in a moderate conserved site, 1 PSS is in a variable site and the other has insufficient data regarding to conservation. Doesn't cluster with other structures.	
<i>M. auratus</i>	Pomc	4	3 PSS are in loops and 1 in a α -helix. 1 is in a variable site and the others have insufficient data regarding to conservation. Clusters with <i>N. galili</i> and <i>P. bairdii</i>	The protein assumes globular conformation in all species, being constituted by α -helices and loops, with exception of the shape of <i>M. pahari</i> species, that shows a more flattened and elongated shape with the presence of an "arm".
<i>M. pahari</i>	Pomc	1	The only PSS is in the protein's "arm" in a variable region in terms of conservation. Doesn't cluster with other structures.	
<i>N. galili</i>	Pomc	3	2 PSS are in loops close to each other and the other is in a α -helix. All have insufficient data regarding to conservation. Clusters with <i>M. auratus</i> and <i>P. bairdii</i>	
<i>P. maniculatus bairdii</i>	Pomc	1	PSS in a α -helix region that is variable in terms of conservation. Clusters with <i>D. ordii</i> , <i>N. galili</i> and <i>M. auratus</i> .	
<i>M. auratus</i>	Per1	10	The 10 PSS are scattered around the protein and have insufficient data regarding to conservation.	The protein assumes globular conformation, being constituted by α -helices and loops.
<i>M. ochrogaster</i>	Fos	3	2 PSS are placed in a bottom part of the protein in a near position to each other, the other in in the upper part of the protein (considering a frontal view). All PSS have insufficient data regarding to conservation.	The protein assumes a curved shape, being mostly made by loops and 3 α -helices
<i>C. porcellus</i>	Il6st	9	The 9 PSS are scattered across the protein, yet 5 PSS are clustered in a small peripheric α -helix structure on the right side (frontal view). All PSS have insufficient data regarding to conservation.	Overall, there is conformational similarity within all structures. The protein structures look like a very elongated "V" and slim and almost look as constituted by
<i>D. ordii</i>	Il6st	4	The 4 PSS are in β -turns and all have insufficient data regarding to conservation.	

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<i>M. auratus</i>	Il6st	5	1 PSS is in a loop, 1 PSS is in a β -strand and the other 3 are in β -turns. All have insufficient data regarding to conservation.	small grouped globular domains made of β -strands and loops, followed in some cases by an "arm" (<i>C. porcellus</i> , <i>D. ordii</i> and <i>M. ochrogaster</i>) mostly made of a main α -helix with loops. Only the <i>R. norvegicus</i> species seems to have more domains that seem to attach to the main elongated structure, forming a more "W" shape.
<i>M. ochrogaster</i>	Il6st	7	5 PSS are in loops, one is in a β -turn and the other in a β -strand. All have insufficient data regarding to conservation.	
<i>R. norvegicus</i>	Il6st	9	All PSS found in the right half of the protein (considering a frontal view), except for 1. 6 PSS are in loops, 2 in β -turns and 1 in a β -strand. 7 PSS are in variable sites and 2 PSS are in conserved sites.	
<i>P. maniculatus bairdii</i>	Ucp4	3	2 PSS are in α -helices and 1 is in a loop. 1 PSS is variable and the other 2 don't have sufficient data regarding to conservation.	The protein presents a channel-like structure
<i>C. porcellus</i>	Col17a1	10	The 10 PSS are scattered around the protein, being 8 in loops and 2 in α -helices. All have insufficient data regarding to conservation.	
<i>D. ordii</i>	Col17a1	4	All the PSS are in loops. All have insufficient data regarding to conservation.	The proteins structures share similarity, looking like an inverted "T" shape with a tilted middle piece. The species having less PSS is a heterotherm (<i>D. ordii</i>) and the one with the most in a hibernator (<i>M. auratus</i>)
<i>M. auratus</i>	Col17a1	16	2 PSS are in β -turns, 1 in a β -strand and the remaining in loops. 2 PSS are in variable sites, and the remaining have insufficient data regarding to conservation.	
<i>O. degus</i>	Col17a1	12	2 PSS are in α -helices and the others are in loops, all scattered around the protein. 2 PSS is in a variable site and the rest have insufficient data regarding to conservation.	
<i>N. galili</i>	Il1r1	6	There are 3 PSS in each extremity of the protein in loops/ β -turns. All have insufficient data regarding to conservation.	The protein in scythe shape with 5 main β -sheets structures paired together with loops at both ends
<i>J. jaculus</i>	Ptges2	2	The 2 PSS are in loops on the upper part of the protein (considering a frontal view) in close position to each other) and both have insufficient data regarding to conservation.	
<i>M. ochrogaster</i>	Ptges2	7	All the 7 PSS are concentrated in one α -helix, sequentially together, with only 1 with a bit more spacing, but still in a very close position to the cluster. All have insufficient data regarding to conservation.	The proteins share globular conformation in both structures, and they are constituted of α -helices, β -sheets and loops.
<i>D. ordii</i>	Ppargc1a	7	The 7 PSS are scattered around the structure, presenting more medial locations, closer to the "hole". 3 PSS are in loops and the other 4 are in α -helices. All have insufficient data regarding to conservation.	The structure presents a globular shape with a "hole" in the centre, possibly to perform some receptor/substrate interaction functions
<i>C. griseus</i>	Clock	2	The 2 PSS are in a loop next to each other in a more peripheric right part of the protein (in a frontal view). All have insufficient data regarding to conservation.	The proteins share globular conformation in all structures, and they are constituted of α -helices and loops.
<i>M. auratus</i>	Clock	1	The PSS is in a loop in a medial-upper position (on a frontal view) close to the hole of the protein and has insufficient data regarding to conservation.	The structures seem all also to share a small internal/central hole, possibly to perform some receptor/substrate interaction functions

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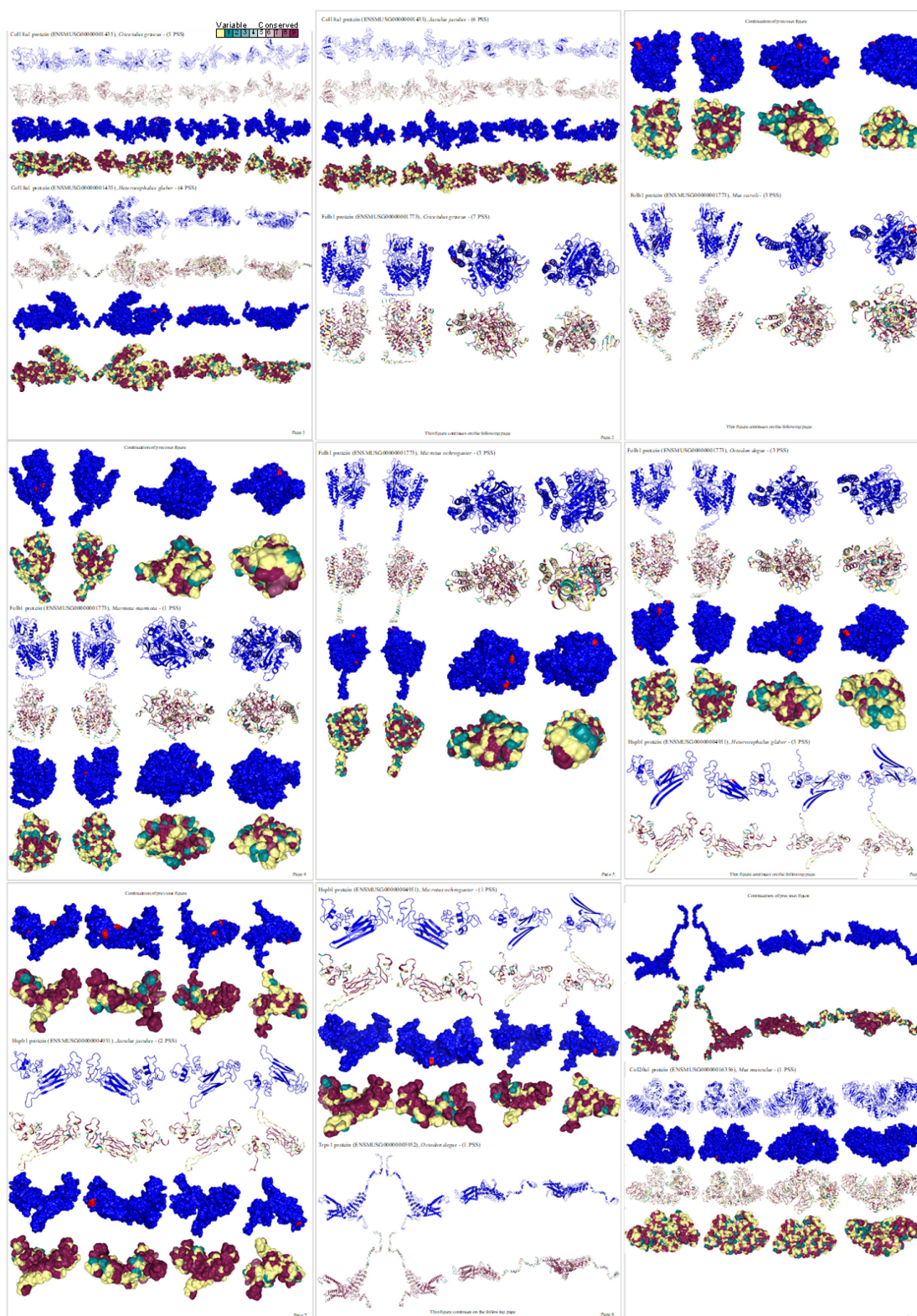
<i>R. norvegicus</i>	Clock	12	When observing the 3rd position – upper view of this structure (in the supplementary data), it is possible to see that the 10 PSS are present in loops, which are concentrated in 3 clusters of the protein. With only 2 PSS being in loops in the bottom part. All PSS have insufficient data regarding to conservation.	
<i>C. porcellus</i>	Anxa4	4	All the 4 PSS assume peripheric/lateral positions, being 3 in α -helices and one in a loop. 1 PSS is in a variable position and the other 3 have insufficient data regarding to conservation.	All the proteins share conformational shape, assuming a globular structure within a "rectangle" (from frontal view). Being all only constituted of α -helices and loops. The species that presented more PSS (<i>M. auratus</i>) is a hibernator.
<i>M. auratus</i>	Anxa4	5	3 PSS are clustered together (2 in a α -helix and 1 in its outing loop); the other 2 PSS are in a α -helix and in a loop, respectively 1 PSS is in a variable position, while the other 4 have insufficient data regarding to conservation.	
<i>M. ochrogaster</i>	Anxa4	1	The only PSS is in a α -helix in an upper part of the protein (considering a frontal view) being in an insufficient site regarding to conservation.	
<i>D. ordii</i>	Stim1	1	The PSS is present in a loop in an upper-right domain, with insufficient data regarding conservation.	
<i>S. vulgaris</i>	Stim1	2	1 PSS is in a α -helix in the bottom part of the protein and the other in an extensive loop region (left side), both considering a frontal view. 1 PSS is in a variable region and the other has insufficient data regarding to conservation.	Both protein structures share a thick "V" like conformation. Being formed only by α -helices and loops.
<i>S. vulgaris</i>	Ano1	5	3 PSS, which are in loops are in the upper part of the protein, being only 1 PSS in a loop on the bottom part (considering a frontal view). The other one is in an extensive "arm" loop. 1 PSS is in a high conserved region, 2 PSS are in a variable position and the other one has insufficient data regarding to conservation.	
<i>H. glaber</i>	Ucp1	5	4 PSS are in upper part of the protein, being 2 of them clustered together on the peripheric left side (frontal view) and only one being in a bottom position in a α -helix. From the upper ones, 2 are in loops and 2 in α -helices. All have insufficient data regarding conservation.	The structures share channel-like conformation. The proteins are formed only by α -helices and loops. This gene only showed Positive selection in African heterothermic species.
<i>J. jaculus</i>	Ucp1	2	1 PSS is in an upper position in a α -helix and the other is almost vertically aligned to it, in a loop, in a bottom position (considering a frontal view). Both have insufficient data regarding conservation.	
<i>P. maniculatus bairdii</i>	Mt2	2	Both PSS are in close positions in loops and have insufficient data regarding conservation.	The protein assumes a very thin curly loop shape with only one small α -helix region
<i>C. porcellus</i>	Mt1	6	All PSS are in loops and have insufficient data regarding conservation.	The protein assumes a very thin curly loop shape
<i>C. porcellus</i>	Trpa1	11	The 11 PSS are mostly concentrated in the left half of the protein (4 in loops and 3 in α -helices) and the others are in loops, on the extremity of the peripheral arm (4 PSS). 1 PSS is in a conserved place, and the other 10 don't have sufficient data regarding to conservation.	The protein shares conformation within species assuming a, elongated spiral shape, with some "arm" structures in one of its extremities. These structures are made from only α -helices and loops.
<i>I. tridecemlineatus</i>	Trpa1	3	2 PSS are in the peripheral arm and 1 in the spiral part, in α -helices and loop, respectively. 2 is in variable regions and the other lacks sufficient data in terms of conservation.	
<i>M. auratus</i>	Trpa1	4	All PSS are in loops, 2 on the peripheral arm and 2 on the spiral part. All have insufficient data regarding conservation.	
<i>O. degus</i>	Trpa1	8	The PSS are scattered in the protein, being 6 in loops and 2 in α -helices. 1 is in a variable site and the others have insufficient data in terms of conservation.	

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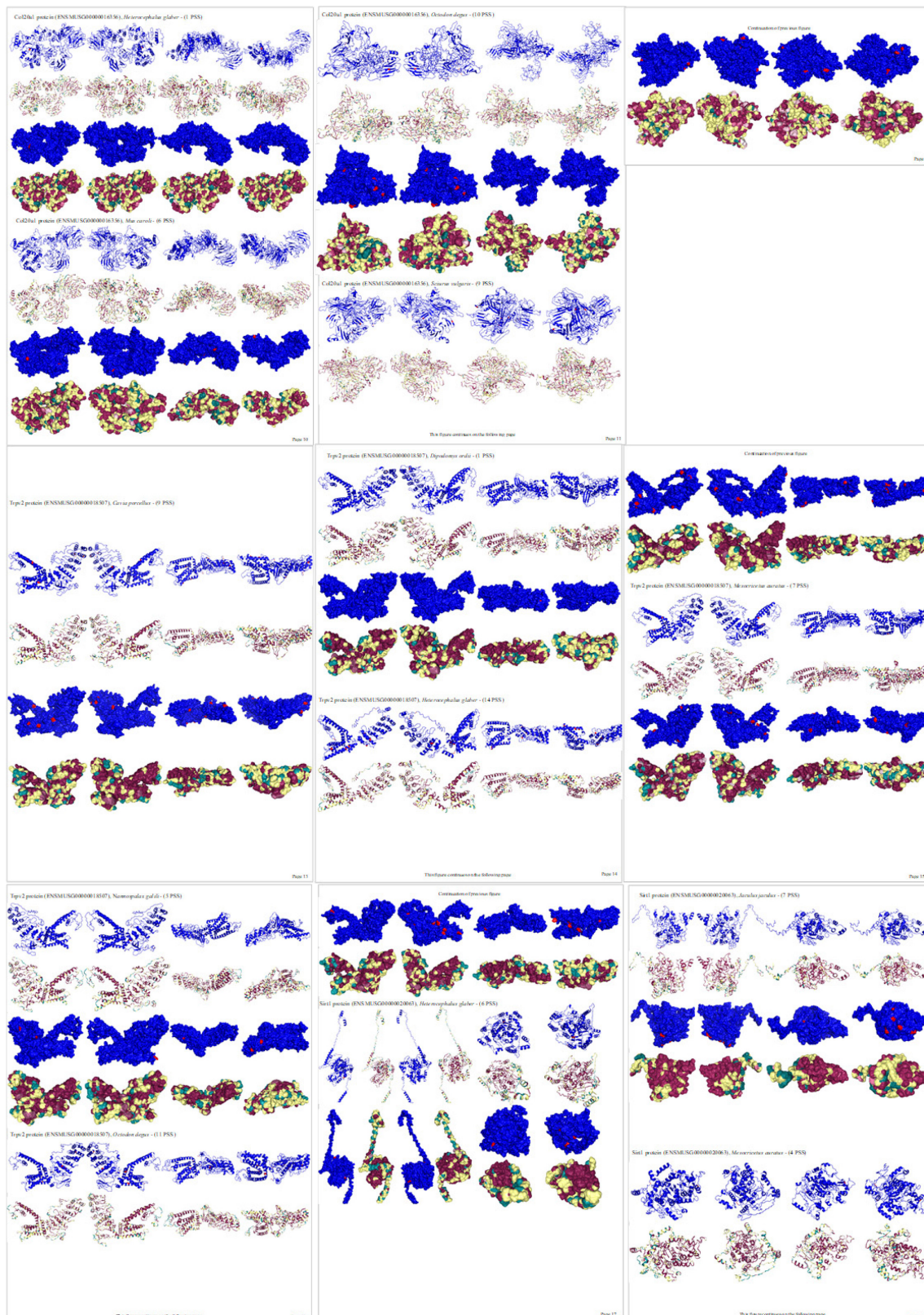
<i>C. griseus</i>	Trpv3	4	The 4 PSS are clustered together in a loop in the upper arm of the protein. All have insufficient data in terms of conservation.	All the structures share a "X" like shape, with some having upper and bottom "arms", except for <i>D. ordii</i> (the only heterothermic spp. showing PSS in this gene) that only has the upper one. The structures are formed by α -helices, 1 β -sheet and loops.
<i>D. ordii</i>	Trpv3	3	2 PSS are clustered together in a α -helix and the other in a loop. All have insufficient data in terms of conservation.	
<i>M. marmota</i>	Trpv3	5	1 PSS is present in a loop while the other 4 are placed in α -helices. 1 has is in a variable region while the others have insufficient data in terms of conservation.	
<i>U. parryii</i>	Trpv3	3	All 3 PSS are in loops, having all insufficient data in terms of conservation.	
<i>N. galili</i>	E2f8	8	The 8 PSS are scattered in the structure, being 6 in loops and 2 in α -helices. All have insufficient data in terms of conservation.	Both structures share a globular shape, being constituted by small scattered α -helices connected by many loops
<i>R. norvegicus</i>	E2f8	6	2 PSS are in α -helices and the remaining in loops. 1 is in a variable site and the others have insufficient data in terms of conservation	
<i>O. degus</i>	Arntl	1	The only PSS is in a loop region with insufficient data regarding the conservation.	The protein is constituted by α -helices, some loops and 2 β -sheet regions that could putatively be ion channels
<i>M. marmota</i>	Per2	13	The 13 PSS are scattered in the structure, being only 4 of them in α -helices and the remaining in loops. All have insufficient data in terms of conservation.	The proteins share an elongated structure, almost looking like a pearled lollipop shape. Being made from a string with several globular loops with a rounded end containing several small α -helices and some β -strands. The proteins share conformation except for the case of <i>M. marmota</i> species which as a completely different shape, assuming a globular shape.
<i>C. lanigera</i>	Per2	6	All the 6 PSS are scattered, 3 PSS are in conserved places and 3 have insufficient data in terms of conservation.	
<i>N. galili</i>	Per2	1	The only PSS is in a loop region with insufficient data regarding the conservation.	
<i>O. degus</i>	Per2	1	The only PSS is in a loop region in a highly conserved region	
<i>J. jaculus</i>	Per2	8	the PSS are mostly in 2 opposite locations of the structure, in which 1 in in a α -helix, 1 in a β -strand, and 6 in loops. All have with insufficient data regarding the conservation.	
<i>I. tridecemlineatus</i>	Per2	9	All the PSS are in loops scattered around the protein. 4 PSS are in conserved places and the others have insufficient data regarding to conservation.	
<i>M. spretus</i>	Tuba1a	18	The PSS are scattered on the structure, being 2 of them in highly conserved regions and the rest in places having insufficient data regarding to conservation.	The structure assumes a globular shape mostly made by α -helices, also including some loops and β -strands

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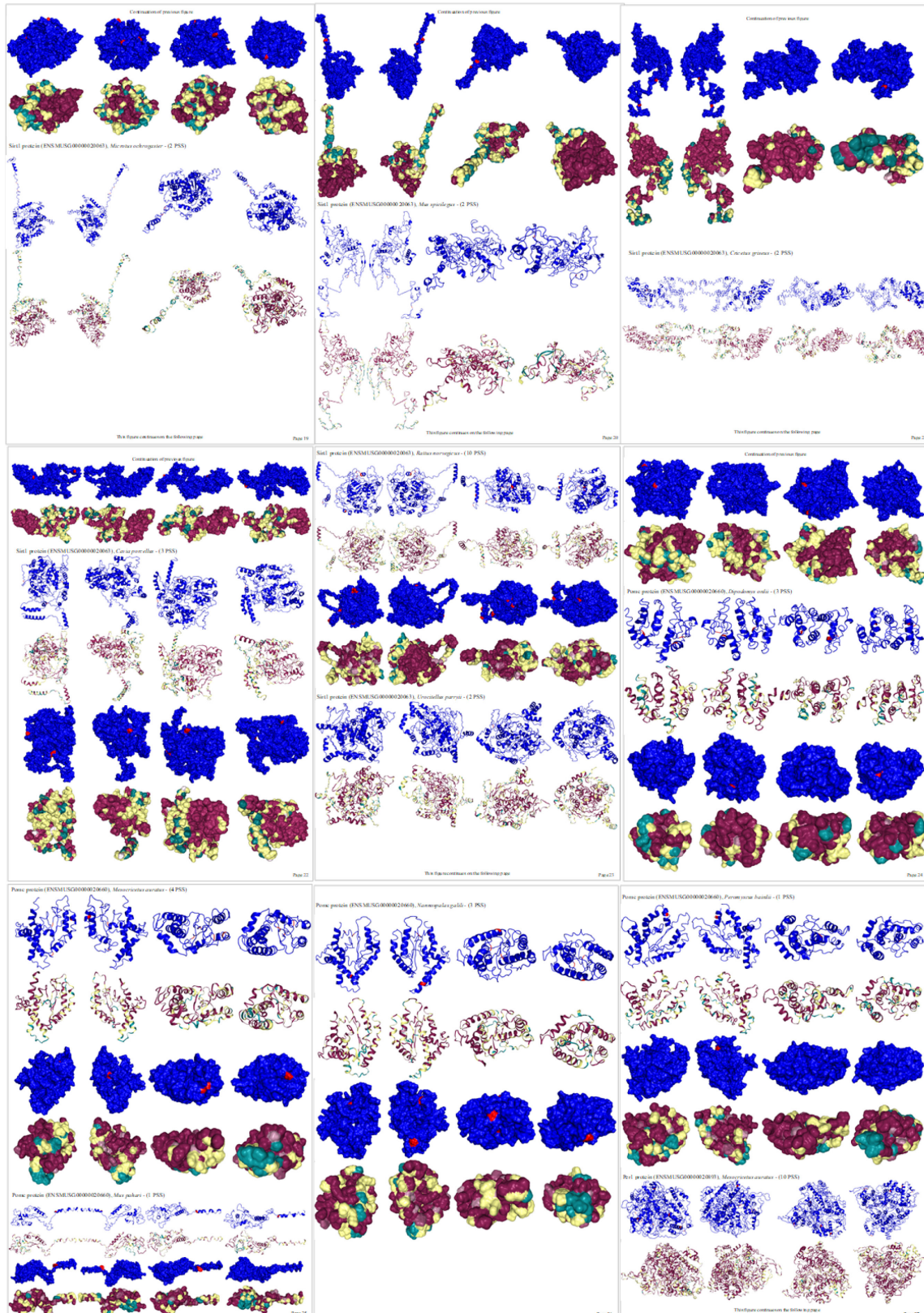
Attachment 7 - supplementary protein structure images, showing PSS data (red) over dark blue coloured proteins; cartoon and surface views, from the same angles) and Consurf functional conservation results according to the colour reference



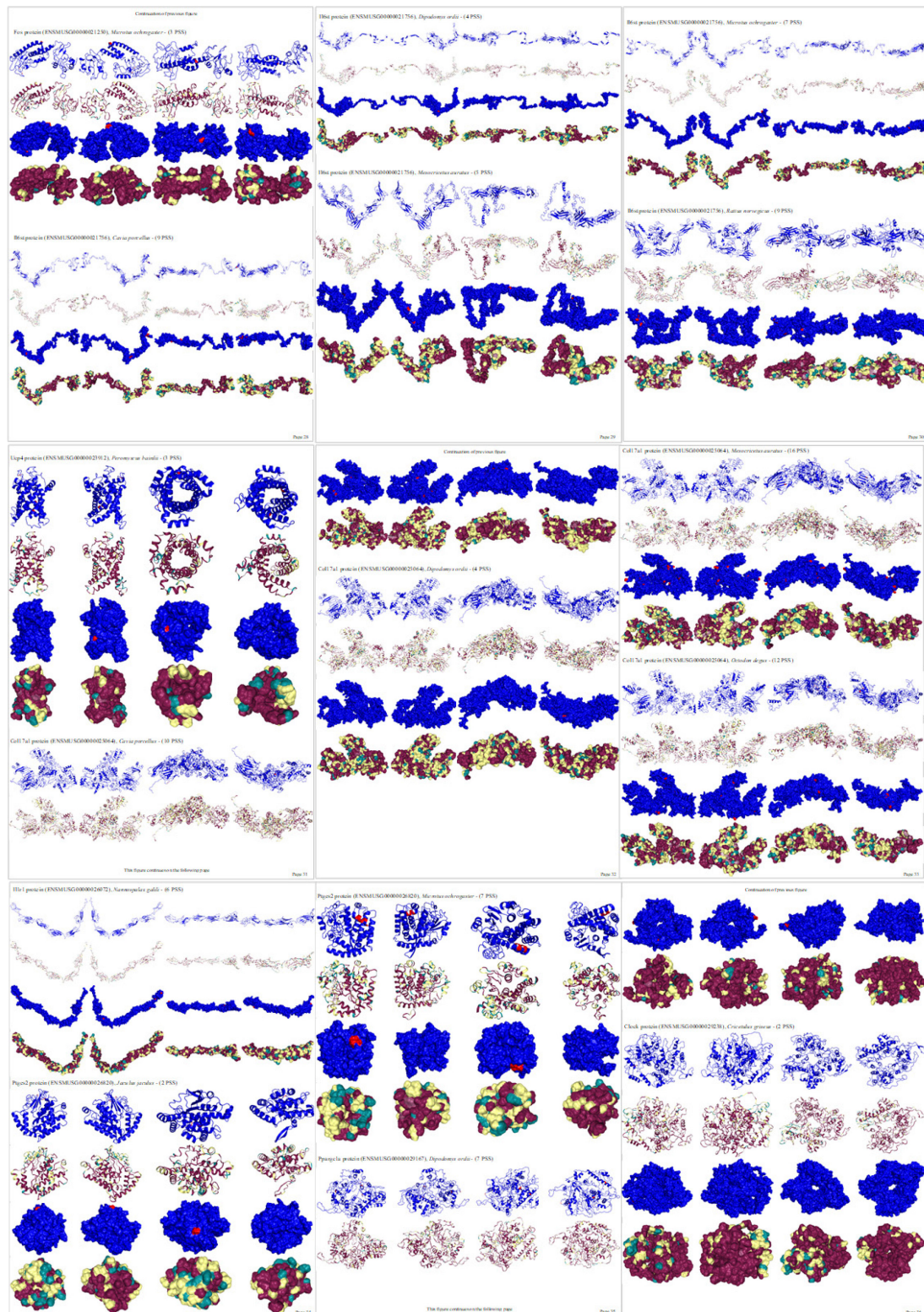
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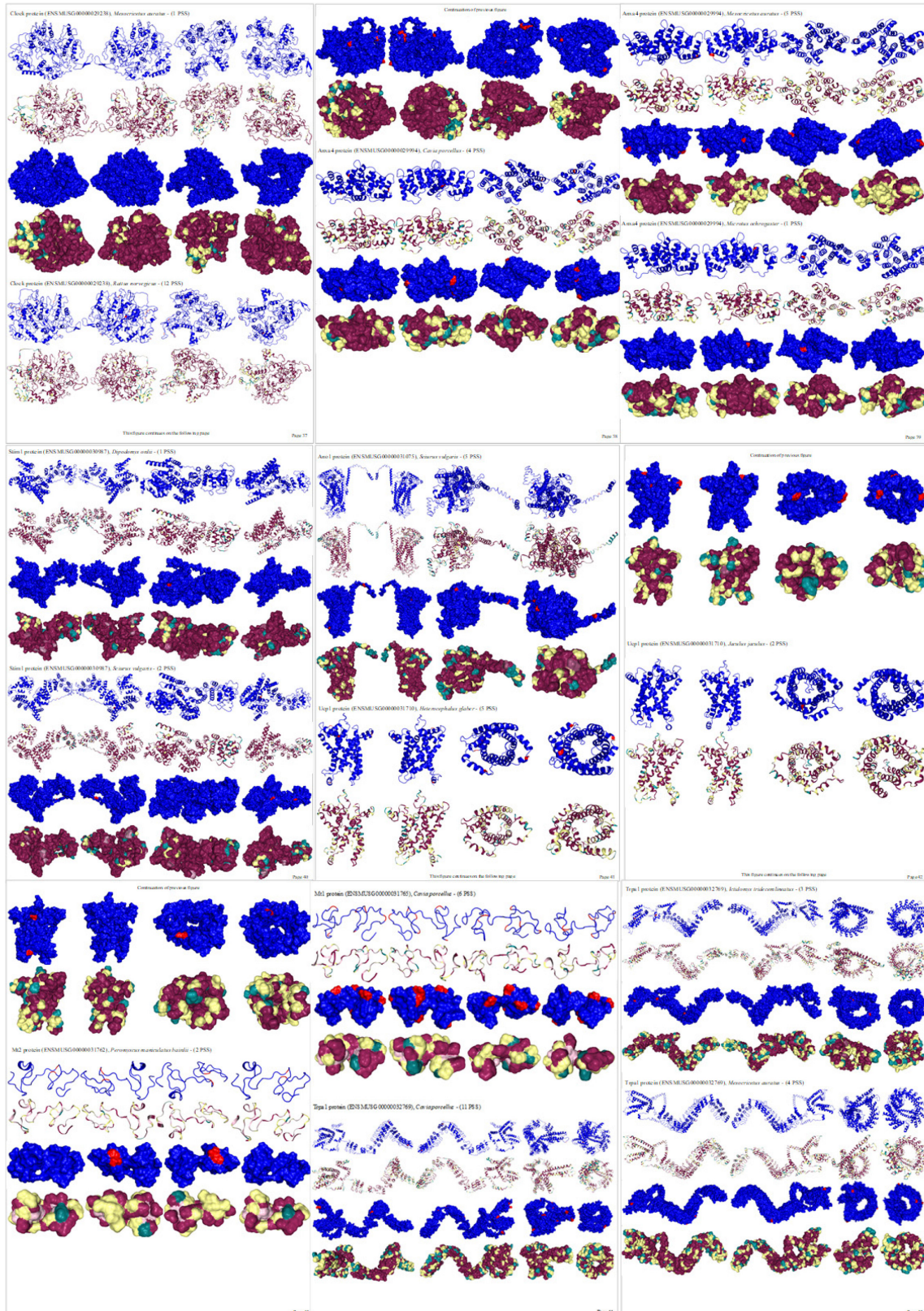
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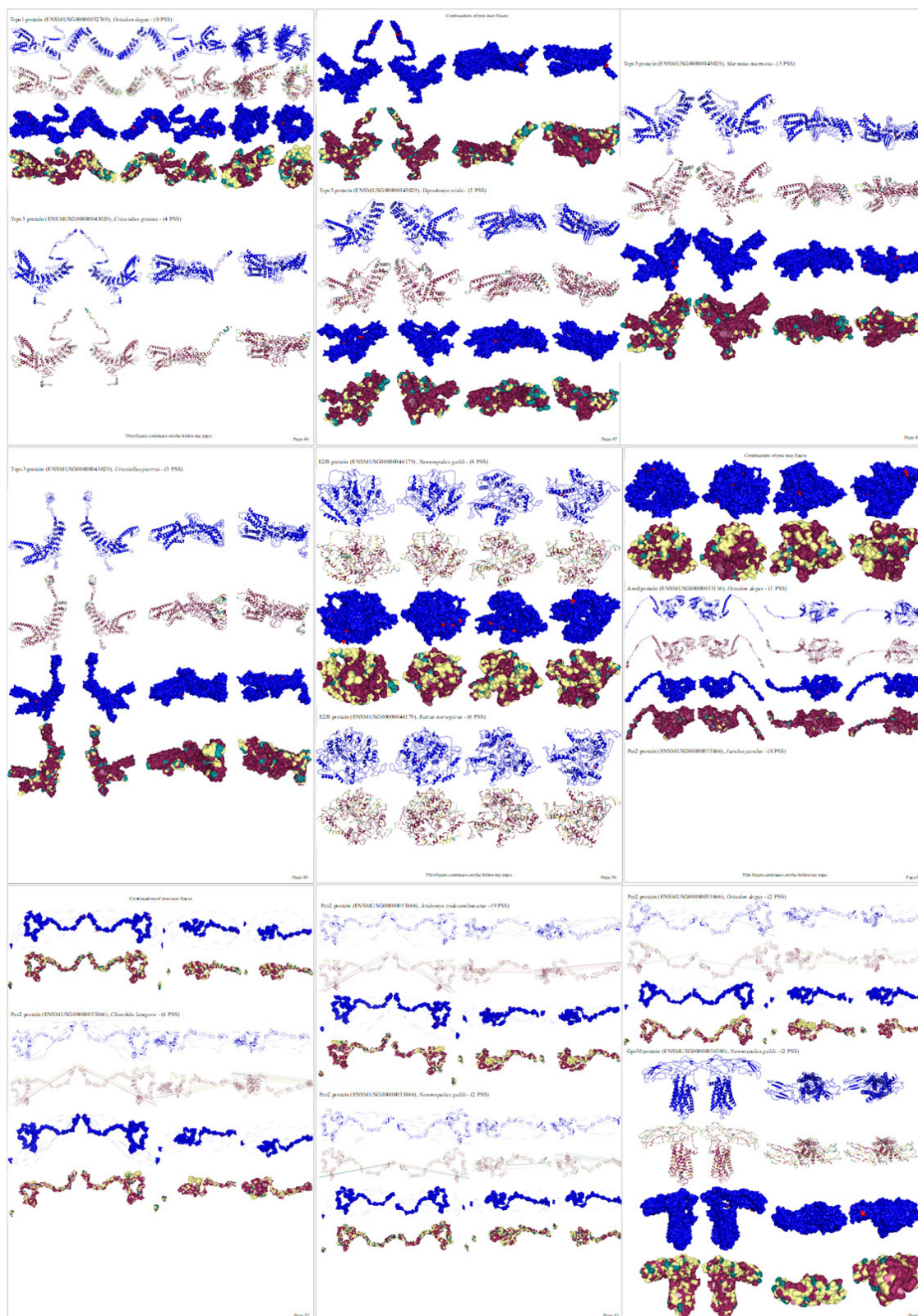
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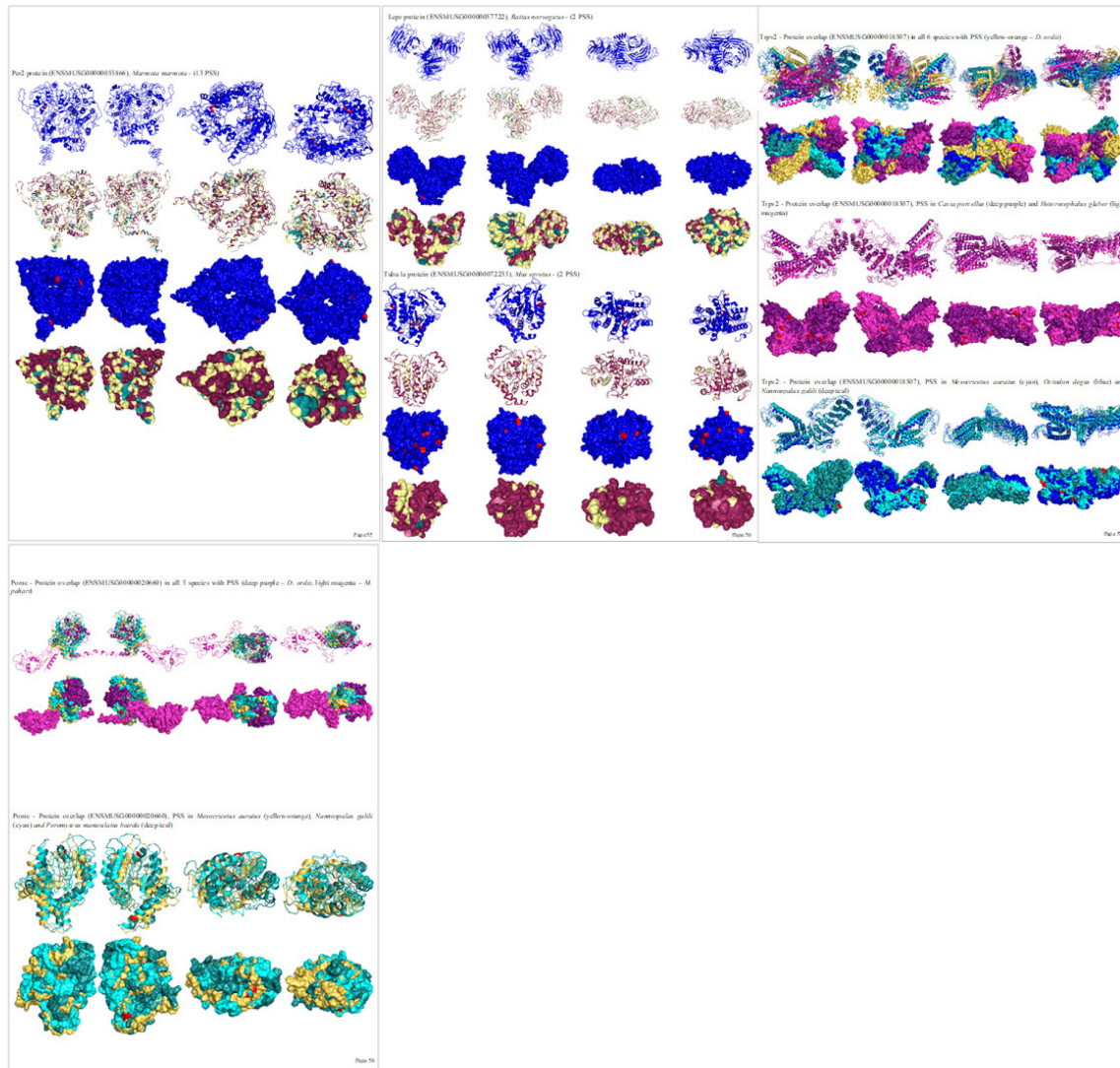
Flexibility of thermoregulation in mammals: Molecular evolution, gene dynamics and ecological drivers



Flexibility of thermoregulation in mammals: Molecular evolution, gene dynamics and ecological drivers



Flexibility of thermoregulation in mammals: Molecular evolution, gene dynamics and ecological drivers



B. Attachments chapter 5

Attachment 9 - Sampling information of specimens from *Jaculus jaculus*, *J. hirtipes* and *G. gerbillus*. The sampling location, date of sampling and the source of data are provided.

Species	Sex	Location	Geographical coordinates (Lat, Long)	Sampling date	Sampling time	Sample ID
<i>Jaculus jaculus</i>	F	Mauritania	16.827125, -16.157737	23.01.2020	01:50:00 AM	D29
<i>Jaculus jaculus</i>	F	Mauritania	16.837957, -16.149825	23.01.2020	11:01:00 AM	D31
<i>Jaculus jaculus</i>	F	Mauritania	19.041812, -15.092960	26.01.2020	11:25:00 PM	D38
<i>Jaculus jaculus</i>	F	Mauritania	19.044415, -15.091852	27.01.2020	10:12:00 AM	D43
<i>Jaculus jaculus</i>	M	Mauritania	20.579422, -16.129780	16.01.2020	02:50:00 AM	D16
<i>Jaculus jaculus</i>	M	Mauritania	20.557157, -16.151753	17.01.2020	03:50:00 AM	D17
<i>Jaculus jaculus</i>	M	Mauritania	16.413418, -16.465935	20.01.2020	04:30:00 AM	D19
<i>Jaculus jaculus</i>	M	Mauritania	19.043818, -15.093622	27.01.2020	12:35:00 AM	D40
<i>Jaculus jaculus</i>	M	Mauritania	19.045007, -15.092515	27.01.2020	01:00:00 AM	D41
<i>Jaculus jaculus</i>	M	Mauritania	23.255317, -15.188707	02.02.2020	04:51:00 AM	D48
<i>Jaculus jaculus</i>	F	Morocco	28.405019, -9.673694	08.01.2020	07:24:00 AM	D12
<i>Jaculus hirtipes</i>	F	Morocco	28.398140, -9.916062	01.01.2020	09:50:00 AM	D2
<i>Jaculus hirtipes</i>	F	Morocco	28.416907, -9.668348	02.01.2020	09:45:00 AM	D7
<i>Jaculus hirtipes</i>	M	Morocco	28.405770, -9.672837	02.01.2020	09:20:00 AM	D6
<i>Jaculus hirtipes</i>	M	Morocco	28.592828, -9.140745	06.01.2020	08:00:00 AM	D11
<i>Jaculus hirtipes</i>	M	Mauritania	16.373815, -16.421493	20.01.2020	04:05:00 AM	D18
<i>Jaculus hirtipes</i>	M	Mauritania	16.399468, -16.434140	20.01.2020	05:00:00 AM	D20
<i>Jaculus hirtipes</i>	M	Mauritania	16.358685, -16.412122	20.01.2020	12:35:00 PM	D22
<i>Jaculus hirtipes</i>	M	Mauritania	16.368080, -16.420075	21.01.2020	02:15:00 PM	D25
<i>Jaculus hirtipes</i>	M	Mauritania	28.377282, -9.995590	03.01.2020	09:57:00 AM	D9
<i>Gerbillus gerbillus</i>	F	Morocco	28.5616234, -11.0106333	17.10.2021	00:05:00	Dg03
<i>Gerbillus gerbillus</i>	F	Morocco	28.6442851, -9.8986267	21.10.2021	01:45:00	Dg18
<i>Gerbillus gerbillus</i>	F	Morocco	28.6364116, -9.9339034	23.10.2021	00:05:00	Dg26
<i>Gerbillus gerbillus</i>	F	Morocco	28.6355731, -9.9348683	24.10.2021	00:30:00	Dg34
<i>Gerbillus gerbillus</i>	F	Morocco	28.5597966, -11.013625	18.10.2021	13:10:00	Dg08
<i>Gerbillus gerbillus</i>	F	Morocco	28.6303867, -9.9324867	22.10.2021	12:10:00	Dg23
<i>Gerbillus gerbillus</i>	F	Morocco	28.63458, -9.9349166	23.10.2021	11:30:00	Dg28
<i>Gerbillus gerbillus</i>	F	Morocco	28.6374567, -9.9345166	23.10.2021	13:25:00	Dg32
<i>Gerbillus gerbillus</i>	F	Morocco	28.6326433, -9.9361617	24.10.2021	12:10:00	Dg40
<i>Gerbillus gerbillus</i>	M	Morocco	28.5562583, -11.018205	18.10.2021	00:00:00	Dg04
<i>Gerbillus gerbillus</i>	M	Morocco	28.5590884, -11.0143213	18.10.2021	00:00:00	Dg05
<i>Gerbillus gerbillus</i>	M	Morocco	28.5616299, -11.0102083	19.10.2021	22:50:00	Dg11
<i>Gerbillus gerbillus</i>	M	Morocco	28.56159, -11.010245	19.10.2021	00:50:00	Dg12
<i>Gerbillus gerbillus</i>	M	Morocco	28.5574616, -11.0158933	19.10.2021	01:20:00	Dg13
<i>Gerbillus gerbillus</i>	M	Morocco	28.6437533, -9.9001867	21.10.2021	01:15:00	Dg17
<i>Gerbillus gerbillus</i>	M	Morocco	28.6326367, -9.9314049	22.10.2021	01:30:00	Dg21
<i>Gerbillus gerbillus</i>	M	Morocco	28.5603983, -11.01354	18.10.2021	13:45:00	Dg09
<i>Gerbillus gerbillus</i>	M	Morocco	28.6338516, -9.9363799	23.10.2021	12:30:00	Dg30

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<i>Gerbillus gerbillus</i>	M	Morocco	28.6327383,-9.9385934	24.10.2021	10:50:00	Dg36
<i>Gerbillus gerbillus</i>	M	Morocco	28.6337334,-9.9363633	24.10.2021	12:30:00	Dg41

Attachment 10 - Number of tissue specific genes identified through differential gene expression analysis using DESeq2.

species	Tissue	total genes (shared readcounts, > 0)	All dif. expresse d genes (padj < 0.05)	cognition DEGs (padj < 0.05)	metabolis m DEGs (padj < 0.05)	% cognition genes	% metabolis m genes	up- regulated cognition	down- regulated cognition	up- regulated metabolis m	down- regulated metabolis m
<i>J. hirtipes</i>	Hippocampus	122424	49	11	6	22.45	12.24	7	4	3	3
	Liver	122424	22	2	6	9.09	27.27	1	1	2	4
<i>J. jaculus</i>	Hippocampus	122424	2415	549	271	22.73	11.22	271	278	123	148
	Liver	122424	58	9	8	15.52	13.79	9	0	7	1
<i>G. gerbillus</i>	Hippocampus	122424	2056	429	254	20.87	12.35	166	263	119	135
	Liver	122424	1615	323	227	20.00	14.06	149	324	109	118
	Total		3671	752	481	* Differential gene expression considering readcount files for the same (overlapping) 122424 genes with > 0 readcounts					

Attachment 11 - Network size variation results across night and day, species and associated genes

species	tissue	genus	nodes night	edges night	net_density night	nodes day	edges day	net_density day	nodes diff	edges diff	net_density diff	total_n	total_e
<i>J. jaculus</i>	Hippocampus	COG	865	30591	0.081864162	2375	7119	0.002525252	1510	-23472	0.07933891	3240	37710
<i>J. hirtipes</i>	Hippocampus	COG	2375	9490	0.003366293	137	427	0.045835122	-2238	-9063	0.04246883	2512	9917
<i>J. jaculus</i>	Liver	COG	2375	11860	0.004206979	2375	11860	0.004206979	0	0	0	4750	23720
<i>J. hirtipes</i>	Liver	COG	2375	44935	0.015939343	2375	17650	0.006260808	0	-27285	0.009678535	4750	62585
<i>G. gerbillus</i>	Hippocampus	COG	2375	2647	0.000938944	2375	4747	0.001683856	0	2100	0.000744912	4750	7394
<i>G. gerbillus</i>	Liver	COG	2375	31006	0.010998448	2375	62234	0.022075644	0	31228	0.011077196	4750	93240
<i>J. jaculus</i>	Hippocampus	ME	415	4891	0.056934986	1528	12188	0.010447203	1113	7297	0.046487783	1943	17079
<i>J. hirtipes</i>	Hippocampus	ME	1528	12188	0.010447203	1528	1710	0.001465763	0	-10478	0.008981441	3056	13898
<i>J. jaculus</i>	Liver	ME	1528	7625	0.006535931	1528	16742	0.014350761	0	9117	0.00781483	3056	24367
<i>J. hirtipes</i>	Liver	ME	1528	27333	0.023429062	1528	4580	0.003925844	0	-22753	0.019503218	3056	31913
<i>G. gerbillus</i>	Hippocampus	ME	1528	7891	0.006763938	1528	16742	0.014350761	0	8851	0.007586823	3056	24633
<i>G. gerbillus</i>	Liver	ME	1528	26141	0.022407314	1528	33989	0.029134394	0	7848	0.00672708	3056	60130

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Attachment 12 - Proportions of positive and negative rewiring (pvalue < 0.01), nodes with positive variation of wTO_sign mean an increased connection during the day

species	tissue	genes	total_count	positive_rewiring_count	proportion_positive	negative_rewiring_count	proportion_negative
<i>G_gerbillus</i>	Hippocampus	COG	2	0	0	0	0
<i>G_gerbillus</i>	Liver	COG	372	13	0.034946237	21	0.056451613
<i>J_hirtipes</i>	Liver	COG	133	0	0	0	0
<i>J_jaculus</i>	Liver	COG	25	0	0	0	0
<i>G_gerbillus</i>	Hippocampus	MET	55	0	0	0	0
<i>G_gerbillus</i>	Liver	MET	463	71	0.153347732	18	0.03887689
<i>J_hirtipes</i>	Hippocampus	MET	8	0	0	0	0
<i>J_hirtipes</i>	Liver	MET	54	0	0	0	0
<i>J_jaculus</i>	Liver	MET	55	0	0	0	0

Attachment 13 - CoDina node classification count results, node proportions and stability/flexibility ratio

species	tissue	genes	alpha_count	gamma_count	beta_count	alpha_prop	gamma_prop	beta_prop	Stability_Flexibility_Ratio
<i>J_hirtipes</i>	Hippocampus	COG	2289	26	60	0.963789474	0.010947368	0.025263	88.03846
<i>J_jaculus</i>	Hippocampus	COG	1854	329	192	0.780631579	0.138526316	0.080842	5.635258
<i>J_hirtipes</i>	Liver	COG	830	836	688	0.349473684	0.352	0.289684	0.992823
<i>J_jaculus</i>	Liver	COG	1022	1138	213	0.430315789	0.479157895	0.089684	0.898067
<i>G_gerbillus</i>	Hippocampus	COG	505	1835	30	0.212631579	0.772631579	0.012632	0.275204
<i>G_gerbillus</i>	Liver	COG	478	1846	25	0.201687764	0.778902954	0.010549	0.258938
<i>J_hirtipes</i>	Hippocampus	MET	1416	22	85	0.929743926	0.014445174	0.055811	64.36364
<i>J_jaculus</i>	Hippocampus	MET	1017	333	171	0.667322835	0.218503937	0.112205	3.054054
<i>J_hirtipes</i>	Liver	MET	317	888	309	0.207596595	0.581532417	0.202358	0.356982
<i>J_jaculus</i>	Liver	MET	443	969	110	0.289921466	0.634162304	0.07199	0.457172
<i>G_gerbillus</i>	Hippocampus	MET	253	1247	22	0.165684348	0.816633923	0.014407	0.202887
<i>G_gerbillus</i>	Liver	MET	165	1324	10	0.108910891	0.873927393	0.006601	0.124622

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Attachment 14 - Results of GO enrichment (BP) for our gene networks (MET and COG) across species and tissues

species	tissue	genes	ID	Description	GeneRatio	BgRatio	RichFactor	FoldEnrichment	zScore	pvalue	p.adjust	qvalue	Count
<i>J_hirtipes</i>	liver	COG	GO:0003008	system process	245/828	528/2356	0.464015	1.320314	6.14969	8.57E-10	7.32E-06	7.10E-06	245
<i>J_hirtipes</i>	liver	COG	GO:0032501	multicellular organismal process	563/828	1413/2356	0.398443	1.133734	5.847983	2.53E-09	1.08E-05	1.05E-05	563
<i>J_hirtipes</i>	liver	COG	GO:0050877	nervous system process	192/828	405/2356	0.474074	1.348935	5.679275	1.52E-08	4.34E-05	4.21E-05	192
<i>J_jaculus</i>	hippocampus	COG	GO:0048513	animal organ development	150/325	778/2356	0.192802	1.397667	5.420478	7.63E-08	0.00048	0.00046	150
<i>J_hirtipes</i>	liver	COG	GO:0007268	chemical synaptic transmission	128/828	260/2356	0.492308	1.400818	5.042974	5.20E-07	0.000640	0.00062	128
<i>J_hirtipes</i>	liver	COG	GO:0098916	anterograde trans-synaptic signalling	128/828	260/2356	0.492308	1.400818	5.042974	5.20E-07	0.000640	0.00062	128
<i>J_hirtipes</i>	liver	COG	GO:0099537	trans-synaptic signalling	128/828	260/2356	0.492308	1.400818	5.042974	5.20E-07	0.000640	0.00062	128
<i>J_hirtipes</i>	liver	COG	GO:0099536	synaptic signalling	130/828	265/2356	0.490566	1.395862	5.034293	5.38E-07	0.000640	0.00062	130
<i>J_hirtipes</i>	liver	COG	GO:0051239	regulation of multicellular organismal process	268/828	619/2356	0.432956	1.231939	4.946153	5.99E-07	0.000640	0.00062	268
<i>J_hirtipes</i>	liver	COG	GO:0007267	cell-cell signalling	163/828	349/2356	0.467049	1.328945	4.900177	9.30E-07	0.000880	0.00085	163
<i>G_gerbillus</i>	hippocampus	COG	GO:0006366	transcription by RNA polymerase II	350/1820	405/2356	0.864198	1.118708	4.836479	3.03E-07	0.001050	0.00105	350
<i>G_gerbillus</i>	hippocampus	COG	GO:0051254	positive regulation of RNA metabolic process	296/1820	339/2356	0.873156	1.130306	4.777041	3.53E-07	0.001050	0.00105	296
<i>G_gerbillus</i>	hippocampus	COG	GO:0141187	nucleic acid biosynthetic process	540/1820	642/2356	0.841121	1.088836	4.861853	4.07E-07	0.001050	0.00105	540
<i>G_gerbillus</i>	hippocampus	COG	GO:0090304	nucleic acid metabolic process	625/1820	750/2356	0.833333	1.078755	4.81263	6.05E-07	0.001050	0.00105	625
<i>G_gerbillus</i>	hippocampus	COG	GO:0006325	chromatin organization	172/1820	190/2356	0.905263	1.171868	4.551889	6.52E-07	0.001050	0.00105	172
<i>G_gerbillus</i>	hippocampus	COG	GO:0006351	DNA-templated transcription	432/1820	508/2356	0.850394	1.100839	4.727805	6.87E-07	0.001050	0.00105	432
<i>G_gerbillus</i>	hippocampus	COG	GO:0006338	chromatin remodelling	155/1820	170/2356	0.911765	1.180284	4.495793	7.80E-07	0.001050	0.00105	155
<i>G_gerbillus</i>	hippocampus	COG	GO:0045893	positive regulation of DNA-templated transcription	280/1820	321/2356	0.872274	1.129164	4.587324	9.84E-07	0.001050	0.00105	280
<i>G_gerbillus</i>	hippocampus	COG	GO:1902680	regulation of RNA biosynthetic process	280/1820	321/2356	0.872274	1.129164	4.587324	9.84E-07	0.001050	0.00105	280
<i>G_gerbillus</i>	hippocampus	COG	GO:0032774	RNA biosynthetic process	520/1820	619/2356	0.840065	1.087468	4.669224	1.10E-06	0.001050	0.00105	520
<i>G_gerbillus</i>	hippocampus	COG	GO:0051252	regulation of RNA metabolic process	444/1820	524/2356	0.847328	1.096871	4.632823	1.17E-06	0.001050	0.00105	444
<i>G_gerbillus</i>	hippocampus	COG	GO:0006355	regulation of DNA-templated transcription	419/1820	493/2356	0.849899	1.100198	4.60921	1.27E-06	0.001050	0.00105	419
<i>G_gerbillus</i>	hippocampus	COG	GO:2001141	regulation of RNA biosynthetic process	419/1820	493/2356	0.849899	1.100198	4.60921	1.27E-06	0.001050	0.00105	419
<i>J_jaculus</i>	hippocampus	COG	GO:0032501	multicellular organismal process	235/325	1413/2356	0.166313	1.20564	4.886578	4.35E-07	0.001360	0.00132	235
<i>G_gerbillus</i>	hippocampus	COG	GO:0006357	regulation of transcription by RNA polymerase II	337/1820	392/2356	0.859694	1.112878	4.509538	1.79E-06	0.001390	0.00139	337
<i>J_hirtipes</i>	liver	COG	GO:0048731	system development	421/828	1044/2356	0.403257	1.147431	4.698085	1.66E-06	0.001410	0.00137	421

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<i>J_hirtipes</i>	liver	COG	GO:0007399	nervous system development	319/828	764/2356	0.417539	1.188071	4.654201	2.29E-06	0.001681	0.00163319
<i>J_hirtipes</i>	liver	COG	GO:0006811	monoatomic ion transport	133/828	278/2356	0.478417	1.361294	4.720726	2.36E-06	0.001681	0.001633133
<i>J_jaculus</i>	hippocampus	COG	GO:0009887	animal organ morphogenesis	75/325	331/2356	0.226586	1.642575	5.043215	1.39E-06	0.00234013	0.0022675
<i>J_jaculus</i>	hippocampus	COG	GO:0009605	response to external stimulus	83/325	379/2356	0.218997	1.587562	4.994045	1.49E-06	0.00234013	0.0022683
<i>G_gerbillus</i>	hippocampus	COG	GO:0045944	positive regulation of transcription by RNA polymerase II	225/1820	256/2356	0.878906	1.137749	4.300782	3.84E-06	0.00278022	0.00278225
<i>J_hirtipes</i>	liver	COG	GO:0055085	transmembrane transport	152/828	328/2356	0.463415	1.318605	4.577247	4.31E-06	0.00283014	0.00274152
<i>G_gerbillus</i>	hippocampus	COG	GO:0016070	RNA metabolic process	543/1820	651/2356	0.834101	1.079749	4.406577	4.31E-06	0.00292066	0.00292543
<i>J_hirtipes</i>	liver	COG	GO:0030154	cell differentiation	381/828	943/2356	0.40403	1.14963	4.366701	8.07E-06	0.00459099	0.00445381
<i>J_hirtipes</i>	liver	COG	GO:0048869	cellular developmental process	381/828	943/2356	0.40403	1.14963	4.366701	8.07E-06	0.00459099	0.00445381
<i>J_hirtipes</i>	liver	COG	GO:0050906	detection of stimulus involved in sensory perception	18/828	22/2356	0.818182	2.328063	4.60605	8.86E-06	0.00472095	0.0045818
<i>J_jaculus</i>	liver	COG	GO:0050877	nervous system process	239/1131	405/2356	0.590123	1.229293	4.871348	7.09E-07	0.00476044	0.00476239
<i>J_jaculus</i>	liver	COG	GO:0003008	system process	302/1131	528/2356	0.57197	1.191477	4.798488	1.01E-06	0.00476044	0.00476302
<i>J_hirtipes</i>	liver	COG	GO:0048856	anatomical structure development	493/828	1262/2356	0.39065	1.111559	4.280303	1.08E-05	0.00490023	0.00475493
<i>J_hirtipes</i>	liver	COG	GO:0015849	organic acid transport	45/828	76/2356	0.592105	1.684783	4.46623	1.17E-05	0.00490023	0.0047545
<i>J_hirtipes</i>	liver	COG	GO:0046942	carboxylic acid transport	45/828	76/2356	0.592105	1.684783	4.46623	1.17E-05	0.00490023	0.0047545
<i>J_hirtipes</i>	liver	COG	GO:0050804	modulation of chemical synaptic transmission	99/828	201/2356	0.492537	1.401471	4.380033	1.20E-05	0.00490023	0.0047599
<i>J_hirtipes</i>	liver	COG	GO:0099177	regulation of trans-synaptic signalling	99/828	201/2356	0.492537	1.401471	4.380033	1.20E-05	0.00490023	0.0047599
<i>G_gerbillus</i>	hippocampus	COG	GO:0045935	positive regulation of nucleobase-containing compound metabolic process	330/1820	386/2356	0.854922	1.106702	4.223575	7.71E-06	0.00492099	0.00492330
<i>J_hirtipes</i>	liver	COG	GO:0042391	regulation of membrane potential	76/828	147/2356	0.517007	1.471097	4.3413	1.58E-05	0.006117	0.0059376
<i>J_hirtipes</i>	liver	COG	GO:0050954	sensory perception of mechanical stimulus	37/828	60/2356	0.616667	1.75467	4.358085	2.01E-05	0.00746013	0.0072337
<i>J_hirtipes</i>	liver	COG	GO:0032502	developmental process	507/828	1308/2356	0.387615	1.102923	4.107564	2.29E-05	0.00816056	0.00791507
<i>J_hirtipes</i>	liver	COG	GO:0007275	multicellular organism development	448/828	1140/2356	0.392982	1.118196	4.088265	2.60E-05	0.00886098	0.00859448
<i>J_hirtipes</i>	liver	COG	GO:0044057	regulation of system process	79/828	156/2356	0.50641	1.440945	4.194541	2.84E-05	0.00907036	0.0087979
<i>J_hirtipes</i>	liver	COG	GO:0022008	neurogenesis	241/828	570/2356	0.422807	1.20306	4.097973	2.96E-05	0.00907036	0.00879241
<i>J_hirtipes</i>	liver	COG	GO:0050974	detection of mechanical stimulus involved in sensory perception	12/828	13/2356	0.923077	2.626533	4.3281	2.97E-05	0.00907036	0.0087912
<i>J_hirtipes</i>	liver	COG	GO:0051241	negative regulation of multicellular organismal process	115/828	244/2356	0.471311	1.341075	4.141392	3.09E-05	0.00911056	0.00883115
<i>J_hirtipes</i>	liver	COG	GO:0048667	cell morphogenesis	110/828	232/2356	0.474138	1.349117	4.121799	3.40E-05	0.00946067	0.00917110

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[illegible]

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[illegible]

Attachment 15 - Results of KEGG enrichment for our gene networks (MET and COG) across species and tissues

species	tissue	genes	category	subcategory	ID	Description	GeneRatio	BgRatio	RichFactor	FoldEnrichment	Score	pvalue	adj.pvalue	qvalue	Count
<i>J_hirtipes</i>	liver	COG	Human Diseases	Substance dependence	mmu05033	Nicotine addiction - Mus musculus (house mouse)	12/547	14/1486	0.857143	2.328545	3.810751	0.001023	0.041419	0.043174	12
<i>J_hirtipes</i>	liver	COG	Environmental Information Processing	Signal transduction	mmu04020	Calcium signalling pathway - Mus musculus (house mouse)	36/547	61/1486	0.590164	1.603261	3.671019	0.000265	0.041917	0.043174	36
<i>J_jaculus</i>	liver	COG	Human Diseases	Endocrine and metabolic disease	mmu04932	Non-alcoholic fatty liver disease - Mus musculus (house mouse)	32/738	40/1486	0.80	1.61084	3.888786	0.020515	0.021542	0.021468	32
<i>J_jaculus</i> <i>J_hirtipes</i> <i>J_hirtipes</i>	hippocampus	COG	Organismal Systems	Digestive system	mmu04974	Protein digestion and absorption - Mus musculus (house mouse)	10/210	18/1486	0.55556	3.931217	5.074213	0.010515	0.011546	0.011458	10

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Attachment 16 – Results of the REML models regarding: $\Delta Ts0_3$, $\Delta Ts3_6$, $\Delta Ts0_6$, $\Delta Tsci_f$, $\Delta Tsci_f$ with sex; and ΔTs , $Ts0$, ΔTsc with $Tsci$ with $Tscidam$

variables tested	fixed effects	Estimate	Std. Error	df	t value	Pr(> t)
$\Delta Ts0_3$	(Intercept)	-4.84311	1.1122	194.656	-4.355	2.15E-05
	ns(P_day,df=3)1	1.63402	0.10337	642.9533	15.807	<2e-16
	ns(P_day,df=3)2	3.75838	0.20938	642.4877	17.95	<2e-16
	ns(P_day,df=3)3	0.96239	0.09031	631.3495	10.656	<2e-16
	mice_photoperiodnight	-0.01084	0.05155	643.363	-0.21	0.83343
	litter_size	-0.01966	0.03042	9.39856	-0.646	0.53353
	TA_exproom	0.1534	0.05631	266.4186	2.724	0.00687
	(Intercept)	-8.35958	1.43371	426	-5.831	1.09E-08
$\Delta Ts3_6$	ns(P_day,df=3)1	4.05727	0.73842	426	5.495	6.75E-08
	ns(P_day,df=3)2	9.99154	2.34752	426	4.256	2.56E-05
	ns(P_day,df=3)3	2.77175	0.45507	426	6.091	2.51E-09
	mice_photoperiodnight	-0.01208	0.04776	426	-0.253	0.800382
	litter_size	-0.04894	0.01875	426	-2.61	0.009375
	TA_exproom	0.15408	0.0462	426	3.335	0.000926
	(Intercept)	-9.39458	1.941754	341.9696	-4.838	1.99E-06
	ns(P_day,df=3)1	6.080127	0.902234	421.0966	6.739	5.26E-11
$\Delta Ts0_6$	ns(P_day,df=3)2	15.96655	2.866474	421.2422	5.57	4.55E-08
	ns(P_day,df=3)3	4.123112	0.557691	422.4558	7.393	7.76E-13
	mice_photoperiodnight	-0.00491	0.059375	426.4111	-0.083	0.934
	litter_size	-0.08122	0.059905	8.335265	-1.356	0.211
	TA_exproom	0.074473	0.069286	360.3684	1.075	0.283
	(Intercept)	-2.87E-01	5.86E-01	2.54E+02	-0.49	0.624
	ns(P_day,df=3)1	1.11E+00	4.80E-02	2.68E+03	23.147	<2e-16
	ns(P_day,df=3)2	4.53E+00	8.39E-02	2.69E+03	53.967	<2e-16
$\Delta Tsci_f$	ns(P_day,df=3)3	5.28E-01	3.89E-02	2.69E+03	13.568	<2e-16
	mice_photoperiodnight	-2.58E-01	2.23E-02	2.69E+03	-11.55	<2e-16

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ΔTsci_f and sex	litter_size	-7.01E-03	2.73E-02	1.03E+01	-0.257	0.803
	TA_exproom	-3.24E-02	2.81E-02	1.38E+03	-1.155	0.248
	(Intercept)	-3.04E-01	5.85E-01	2.55E+02	-0.52	0.604
	ns(P_day,df=3)1	1.13E+00	4.78E-02	2.68E+03	23.661	<2e-16
	ns(P_day,df=3)2	4.52E+00	8.36E-02	2.69E+03	54.103	<2e-16
	ns(P_day,df=3)3	5.32E-01	3.86E-02	2.69E+03	13.777	<2e-16
	sexM	3.95E-03	2.42E-02	2.47E+03	0.163	0.87
	mice_photoperiodnight	-2.62E-01	2.22E-02	2.69E+03	-11.776	<2e-16
	litter_size	-6.02E-03	2.69E-02	1.02E+01	-0.224	0.827
	TA_exproom	-3.21E-02	2.81E-02	1.33E+03	-1.144	0.253
ΔTs and Tscidam	(Intercept)	0.490352	1.720163	356.7899	0.285061	0.775763
	ns(P_day,df=3)1	0.333467	0.081654	1055.396	4.083901	4.76E-05
	ns(P_day,df=3)2	1.512635	0.125429	1054.493	12.0597	1.82E-31
	ns(P_day,df=3)3	0.183524	0.063626	1057.406	2.884421	0.004001
	Tscidam	0.018093	0.036608	874.3076	0.494229	0.621268
	mice_photoperiodnight	0.114187	0.040691	1002.315	2.806203	0.00511
	litter_size	-0.04931	0.036953	8.467591	-1.33444	0.21683
	TA_exproom	-0.06842	0.055912	155.311	-1.22379	0.222887
	(Intercept)	25.85126	2.258346	418.1663	11.44699	1.40E-26
	ns(P_day,df=3)1	-2.03152	0.099561	1055.179	-20.4049	2.86E-78
Ts0 and Tscidam	ns(P_day,df=3)2	-3.27512	0.153904	1055.814	-21.2803	6.64E-84
	ns(P_day,df=3)3	-1.4889	0.078251	1059.939	-19.0271	1.14E-69
	Tscidam	0.086106	0.045622	1058.577	1.887384	0.059382
	mice_photoperiodnight	-0.1477	0.050234	1062.779	-2.94028	0.00335
	litter_size	0.266807	0.083233	8.245489	3.205559	0.012021
	TA_exproom	0.032235	0.074142	669.0409	0.434776	0.663865
	(Intercept)	1.719085	1.707757	460.6391	1.006633	0.31464
	ns(P_day,df=3)1	2.298953	0.076306	1055.957	30.12822	2.03E-144
	ns(P_day,df=3)2	2.804078	0.117951	1056.614	23.7733	2.05E-100
	ns(P_day,df=3)3	2.11575	0.059951	1061.064	35.29133	4.14E-181
ΔTsc and Tscidam	Tscidam	-0.13757	0.034903	1050.992	-3.94145	8.63E-05
	mice_photoperiodnight	0.010329	0.038455	1061.171	0.268595	0.788294
	litter_size	-0.03489	0.056619	8.547076	-0.61629	0.553757
	TA_exproom	0.139387	0.056258	550.498	2.477626	0.013525
	(Intercept)	32.94813	1.610031	506.4466	20.46428	2.80E-68
	ns(P_day,df=3)1	0.846072	0.068601	1263.639	12.33325	4.41E-33
	ns(P_day,df=3)2	1.476438	0.104344	1263.411	14.14966	2.66E-42
	ns(P_day,df=3)3	0.569707	0.053544	1267.069	10.64002	2.20E-25
	Tscidam	0.075732	0.032211	1242.846	2.351154	0.018871
	mice_photoperiodnight	0.166977	0.032506	1268.029	5.136759	3.23E-07
Tsci and Tscidam	litter_size	-0.14599	0.051549	9.381255	-2.83205	0.01889
	TA_exproom	0.04246	0.053432	572.3083	0.794643	0.42715