Waking the sleeping dragon: molecular insights into the hibernation of the central bearded dragon

by

Alexander Capraro

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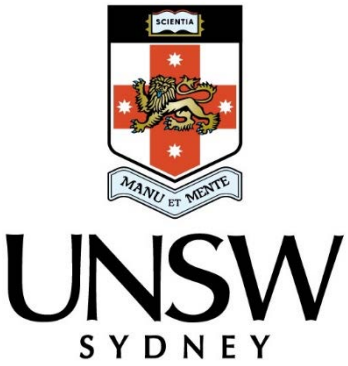
Biochemistry and Molecular Genetics



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Abstract:

One of the most profound adaptive strategies employed by animals in response to changes in environmental conditions is hibernation. Hibernation is an extended state of hypometabolism and dormancy induced to survive the low food availability and high physiological stress associated with winter. During the long periods of dormancy, hibernators experience changes in core biological processes, including drastic reductions in basal metabolic rate, core body temperature, oxygen consumption, heart rate and brain activity. Stress response pathways are also modulated to mitigate physiological challenges that would otherwise prove lethal to the animal. Modulation of these responses requires tightly controlled regulatory processes. While hibernation is a common response utilised by diverse lineages of animals, molecular investigations into this phenomenon have primarily focused on mammalian systems.

In this thesis, I investigated the molecular mechanisms involved in the hibernation of the Australian central bearded dragon (*Pogona vitticeps*) using high-throughput sequencing technologies. The transcriptomic and proteomic changes in three tissues; brain, heart and skeletal muscle, during hibernation and at two time points post-arousal were examined. Hibernation was associated with the induction of common and tissue-specific stress response pathways in addition to the modulation of regulatory processes. I studied the role of microRNAs (miRNA) in mediating the changes in gene expression that are characteristic of the three tissues during hibernation and four days post-arousal. Cellular metabolism and neuroprotection of the brain emerged as key pathways under miRNA-mediated regulation. The DNA methylation dynamics in brain and skeletal muscle during hibernation and at two time points post-arousal were also examined. Alterations in DNA methylation were associated with changes in gene expression that lead to increased neuroprotection of the brain and a reduced potential for atrophy in skeletal muscle. Collectively, the data presented in this thesis shows that the precise interplay of multiple gene regulatory mechanisms is crucial in modulating the large-scale changes in cellular physiology and function observed in hibernating bearded dragons. Furthermore, the data presented bridges the gap between mammalian and reptilian hibernation and suggests they are more similar than previously thought.

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List of abbreviations

2Dv2M	Two days post-arousal versus two months post-arousal
3C	Chromosome conformation capture
5caC	5-carboxycytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATAC	Ada2a-containing
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
A-to-I	Adenosine-to-inosine
BER	Base excision repair
BMP	Bone morphogenetic protein
bpm	Beats per minute
BS-seq	Bisulfite sequencing
CAB-seq	Chemical modification-assisted bisulfite sequencing
CGI	CpG islands
ChIP-seq	Chromatin immunoprecipitation sequencing
CpG	Cytosine-phosphate-guanine
CPM	Counts per million
CT	Circadian time
CTE	Chronic traumatic encephalopathy
DEG	Differentially expressed gene

DEmiR	Differentially expressed miRNA
DMG	Differentially methylated gene body
DMP	Differentially methylated promoter
DMR	Differentially methylated region
DNMT	DNA methyltransferase
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FFA	Free fatty acid
G_1	Gap phase 1
G_2	Gap phase 2
GO	Gene Ontology
GSEA	Gene set enrichment analysis
HDAC	Histone deacetylase
Hv2D	Hibernation versus two days post-arousal
Hv2M	Hibernation versus two months post-arousal
IBA	Interbout arousal
IPC	Ischemic preconditioning
iTRAQ	Isobaric tags for relative and absolute quantification
kb	Kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
m^1A	N1-methyladenosine
m^6A	N^6-methyladenosine
MAB-seq	Methylase-assisted bisulfite sequencing

MAPT	Microtubule associated protein tau
Mb	Megabase
MBD	Methyl-CpG-binding domain
miRNA	MicroRNA
mRNA-seq	mRNA sequencing
MSTN	Myostatin
NFT	Neurofibrillary tangle
NMDA	N-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
oxBS-seq	Oxidative bisulfite sequencing
PHF	Paired-helical-filament
piRNA	Piwi-interacting RNA
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RRBS	Reduced representation bisulfite sequencing
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SNP	Single nucleotide polymorphism
SUMO	Small ubiquitin-like modifier
T_b	Core body temperature
TCA	Tricarboxylic acid

TET	Ten-eleven translocation
TGF-β	transforming growth factor beta-receptor
TMM	Targeted temperature management
TMT	Tandem mass tags
TSS	Transcription start site
UTR	Untranslated region
WGBS	Whole genome bisulfite sequencing
ZT	Zeitgeber time

1 Introduction

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1.1 Scope of thesis

In this thesis I examine the molecular mechanisms involved in hibernation of the Australian central bearded dragon (*Pogona vitticeps*) – hereinafter referred to bearded dragon. Hibernation is a common strategy throughout the animal kingdom that allows organisms to survive the harsh environmental conditions associated with winter. Hibernators reduce energy-intensive processes and induce stress responses. Physiological and cellular features of hibernation are conserved throughout eukaryotes, however, molecular-based research into hibernation is mainly focused on mammalian systems. Reptiles, such as the bearded dragon, possess a remarkable capacity to withstand stress associated with hibernation and provide a perfect system to study non-mammalian hibernation.

The first study examined changes of gene expression in the transcriptome (RNA-seq) and proteome (mass-spectrometry) to determine which metabolic pathways were altered during hibernation. The second examined epigenetic role of miRNAs in hibernation through small RNA-seq. The final study investigated the potential role of DNA methylation in regulating gene expression, using reduced representation bisulfite sequencing (RRBS). The data presented in this thesis provides evidence for large scale changes in gene expression that lead to induction of key adaptive and stress responses in a global and tissue-specific manner.

This project had two main aims:

1. Discover metabolic pathways and changes in gene expression that are involved in central bearded dragon hibernation

2. Uncover the epigenetic regulatory mechanisms that lead to the hibernation phenotype.

1.2 Chapter summary

In **Chapter 1** I reviewed the literature of hibernation. This is largely from the context of what is known in mammals, including the biological processes that are modulated during hibernation and the roles of several regulatory processes. I contrast reptilian hibernation with mammalian hibernation, highlighting the need for research in non-mammalian hibernators. Finally, I examine how high-throughput technologies unlock an unprecedented ability to study biological systems, and what can be examined when implemented in hibernation research.

In **Chapter 2**, using RNA-seq and protein mass spectrometry, I investigated the changes in mRNA and protein expression of the brain, heart and skeletal muscle at three time points: late hibernation, two days post-arousal, and two months post-arousal. Large-scale differential mRNA and protein expression was observed. Subsequent biological pathway analysis permitted a tissue-specific understanding of the stress responses and changes in cellular metabolism that are involved in bearded dragon hibernation.

In **Chapter 3**, using small RNA-seq, I examined the role of microRNAs in regulating key biological processes in the brain, heart and skeletal muscle during hibernation. My results support the hypothesis that miRNAs are key regulators of genes involved in metabolic changes organism-wide, and increased neuroprotection in the brain.

In **Chapter 4**, using RRBS, I assessed the role of DNA methylation in regulating mRNA expression during hibernation in brain and skeletal muscle. I identified differentially methylated gene bodies and promoters that correlated with RNA-seq data. This revealed

that neuroprotection of the brain and prevention of muscle atrophy were guided by tissue-specific changes in DNA methylation.

In **Chapter 5**, I summarise the results of my research chapters, and discuss their contribution to understand the differences and similarities between mammalian and reptilian hibernation. I provide insight into using hibernation as a resource for biomedical research and suggest avenues for future research in hibernation.

1.3 Hibernation

Many organisms possess unique abilities to alter their physiological state in response to deleterious environmental conditions to increase the likelihood of survival. One of the most well-known and drastic physiological adaptive strategies is hibernation. Hibernation is a seasonal state of dormancy and metabolic depression that allows animals to survive weeks or even months of physiological stress associated with the cold winter months where food availability is scarce. Hibernation is utilized by diverse species of mammals, reptiles, amphibians and birds [1, 2]. However, most of what is known about hibernation is restricted to mammals. While some may debate the use of the term hibernation in reptiles, in this season I chose to refer to seasonal metabolic depression in reptiles as hibernation. In later chapters, I will avail myself of a model of a hibernation continuum as well as the similarities towards mammalian hibernation that will support this decision.

During these long periods of dormancy, mammalian hibernators experience drastic changes in cellular metabolism, biochemical function, key physiological processes, and even behaviour [3]. The precise alterations experienced by hibernators vary from species to species. Rather than there being strict physiological responses experienced by all hibernators, the responses exist on a continuum [4]. For decades “true” hibernators were thought to only include mammals that severely and significantly decrease their metabolic rates and core body temperature (T_b) such as the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*). However, recent research may suggest a more general definition, that is a physiological process that involves significant reductions in metabolic rate and T_b for an extended period during winter. Under this definition, hibernators can experience

various degrees of reduction in basal metabolic rate, oxygen consumption, heart rate, T_b, transcription and translation rates, and depressed immune responses.

Being in a state of metabolic depression exposes the organism to a host of stressors, including inadequate nutrition, oxidative stress, hypoxia, ischemia (restricted blood flow), reperfusion injury (return of blood flow after ischemia), dehydration, and immune challenge. In order to mitigate these challenges, a variety of metabolic pathways must be modulated. This requires a regulatory system that can specifically and precisely alter expression of key genes that modulate these protective pathways. In mammals there is clear evidence of gene expression control that ranges from transcription to post-translation [5].

1.4 Physiological changes associated with hibernation

The hibernation phenotype is associated with large scale changes that lead to an overall restructuring of cellular, tissue, and organ physiology. These deviations from euthermia result in a net reduction in ATP turnover, reprioritisation of cellular processes to minimise fuel expenditure, stabilisation of cellular machinery, and induction of stress responses to ensure long-term viability and function. Furthermore, tissue- and organ-specific changes are employed to ensure their maintenance of function and survival.

1.4.1 Torpor cycles

The hibernation period of mammals typically involves long periods of dormancy and metabolic depression, known as torpor, that are interspersed with shorter periods of arousal, known as interbout arousal (IBA; **Figure 1-1**). When animals enter IBAs, normal body temperatures (euthermia) and metabolic rates return. The extended periods of torpor

undeniably reduce energy expenditure, however, the reason for the energy intensive return to euthermia is not known. Due to the large energy costs and the precise timing involved in IBAs, it is presumed to be crucial for hibernation in many mammals.

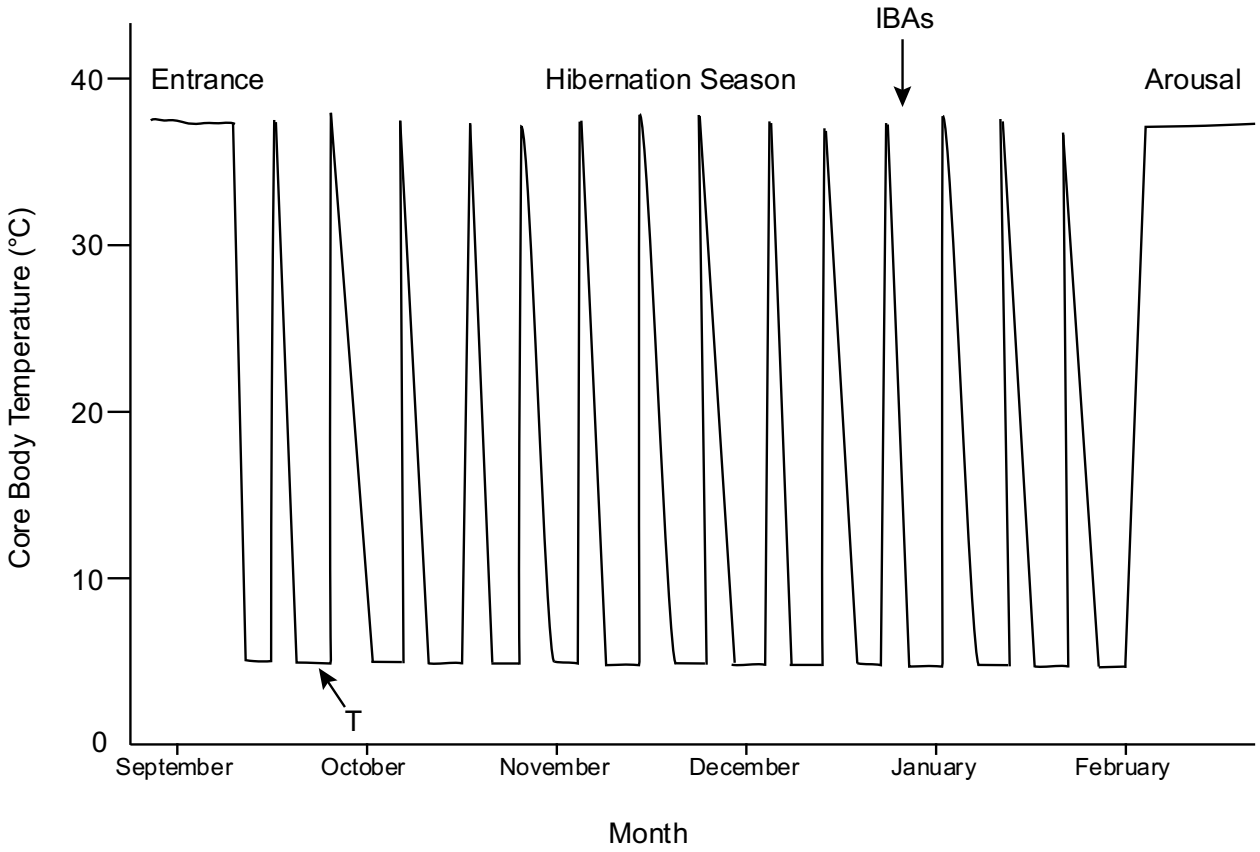


Figure 1-1. Temperature fluctuations experienced throughout the hibernation season in a mammal. Periods of torpor (T) are interspaced with periods of interbout arousal (IBA).

Gene transcription and protein synthesis are re-established both passively and actively during IBAs [6, 7]. Moreover, gene expression appears to be over activated during IBAs as transcription initiation rates are roughly 20% higher during IBAs than during euthermia in ground squirrels [7]. This suggests that IBAs may serve an important protective process that allows for critical gene and protein products to be maintained and restocked throughout hibernation. Furthermore, there is evidence for resumption of inflammatory and immune responses, in addition to recovery of synapses during IBAs [8].

1.4.2 Depression of energy-expensive metabolic processes

The main purpose of this reduced metabolic rate is to conserve energy, allowing for animals to survive weeks or months of anorexia. ATP-demanding, but unessential, metabolic processes are depressed during hibernation, with energy diverted to essential processes.

In the ground squirrel hibernation model, metabolic processes subject to depression include: global gene transcription [7], protein translation and synthesis [6, 9], cell cycle progression [10] and transmembrane ion transport [11]. As gene transcription can account to up to 10% of the energy demand of mammalian cells [12], it is unsurprising that global gene transcription is depressed. In ground squirrels, transcription initiation is approximately 60% lower than during euthermia, with elongation almost non-existent [7]. However, total mRNA abundance is unaffected [9, 13]. Much like transcription, translation is also energy expensive. In the brain of hibernating ground squirrels, translational rates are less than 1% of that of euthermic brains [9]. Translational initiation is halted during entrance into hibernation preventing new peptides being formed, and pre-initiated polysomes are extended three times slower than during euthermia [6, 9].

Cell cycle activity is a metabolic pathway that is particularly ATP demanding. The cell cycle consists of a series of phases with checkpoints to ensure proper DNA replication and subsequent cell division (mitosis). Cells that have reversibly halted the cell cycle are known as quiescent. Typically, the cell cycle can be reversibly halted at two main checkpoints: gap phase 1 (G_1), where sufficient growth and DNA damage is checked to ensure proper DNA replication; and gap phase 2 (G_2), where DNA replication is checked to have occurred correctly [14]. Progression through these checkpoints is primarily

mediated by cyclin proteins, which function by binding and activating cyclin-dependant kinases (CDKs) [15].

CDKs are a major regulatory for cell cycle progression. Together with cyclin binding, reversible phosphorylation of CDKs is necessary for complete activation [14]. Competitive binding of CDKs with CDK inhibitors such as p15 and p21 or prevention of reversible phosphorylation allows for inhibition of cyclin-CDK complexes [14]. In hibernators, protein and mRNA levels of cyclins are downregulated by up to 50% during torpor in liver [10]. Furthermore, p15 and p21 are upregulated 1.4- and 1.7 to 2.0-fold, respectively. These indirect observations suggest that cells are quiescent during hibernation, potentially reflecting a means to reduce total energy expenditure.

Collectively, limiting the initiation and elongation of transcription and translation, and prevention of cell cycle progression would reduce energy expenditure of hibernators dramatically. However, the most energy-intensive process in most cells is ion transport across the cell membrane. ATP-driven ion pumps allow for ions such as sodium (Na^+), potassium (K^+), hydrogen (H^+) and calcium (Ca^{2+}) to be moved against the concentration gradient. This facilitates maintenance of membrane potential and homeostasis in all cells, neurotransmission in neurons, and contraction in muscles. This energy intensive process can be responsible for 25% of ATP use in human kidney cells, 50% in human erythrocytes, and up to 70% in neurons [16, 17]. In skeletal muscle, the sodium pump (Na^+ K^+-ATPase) accounts for more than 20% of total energy consumption alone [18]. As such, it is unsurprising that in skeletal muscle, kidney and liver of hibernating ground squirrels Na^+ K^+-ATPase activity drops by 40–60% compared to euthermic animals [11].

1.4.3 Shifts in cellular metabolism

The lack of food consumption during hibernation requires shifts in fuel sources to properly utilise stored energy. Prior to hibernation, hibernators typically store large amounts of triglycerides in adipose tissue. This permits hibernators to switch the major fuel source from glucose to stored lipids and triglycerides. Large amounts of fat is stored in adipose tissue over the months prior to hibernation [19]. These molecules are metabolised via four main processes: 1) lipolysis; 2) beta-oxidation; 3) ketogenesis; and 4) gluconeogenesis (**Figure 1-2**).

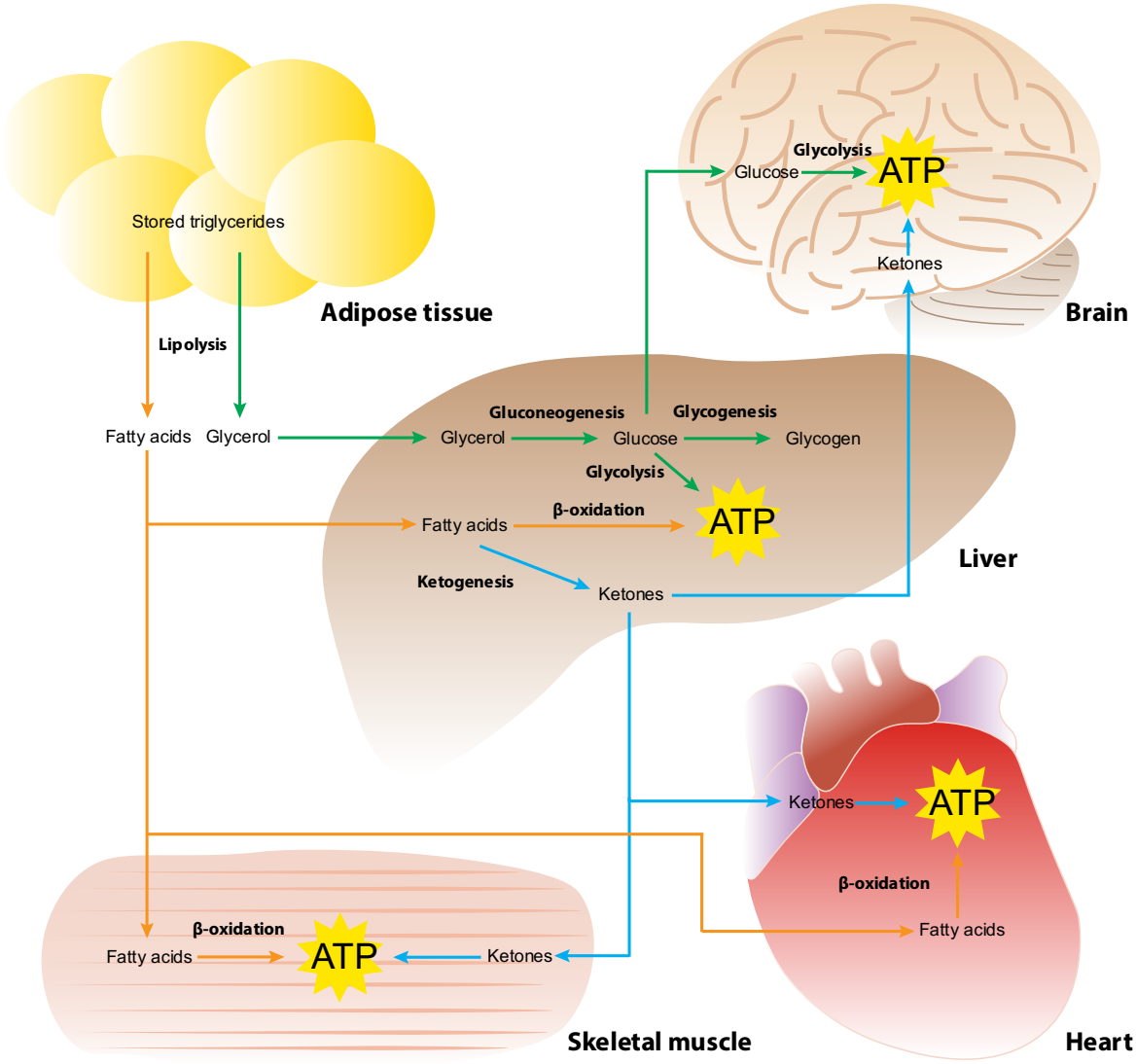


Figure 1-2. Cellular metabolism during hibernation in a mammal. Hibernation involves the switch from glucose-based metabolism to metabolism of stored triglycerides. Triglycerides are metabolised via lipolysis into fatty acids (orange arrows), where they can be utilised by all organs but the brain, and glycerol (green arrows). Fatty acids are metabolised via β-oxidation into acetyl-CoA which is fed into the TCA cycle to produce ATP. Glycerol is metabolised into glyceraldehyde-3-phosphate, which enters the gluconeogenic pathway to produce glucose. Glucose can be used to produce ATP via glycolysis or used to replenish glycogen stores in the liver. Alternatively, acetyl-CoA produced from fatty acids can be converted into ketones (blue arrow) via ketogenesis, which can be transported through the blood brain barrier into the brain. Ketones are converted back into acetyl-CoA to enter the TCA cycle.

During hibernation, stored triglycerides are hydrolysed via lipases to produce free fatty acids (FFAs) and glycerol [20] (**Figure 1-2**). The glycerol separated from the FFAs allows it to be transported throughout the body via the blood. Long-chain FFAs are metabolised by the mitochondria through β-oxidation two carbon molecules at a time; producing one acetyl-CoA molecule and 5 ATP molecules [21]. Acetyl-CoA is fed into the tricarboxylic acid (TCA) cycle, ultimately producing 1 GTP and 11 ATP molecules per acetyl-CoA molecule.

As the brain cannot utilise FFAs as an energy source, liver acetyl-CoA is diverted to form the ketone bodies acetoacetate and β-hydroxybutyrate through a process called ketogenesis [22] (**Figure 1-2**). Ketone bodies can pass through the blood-brain barrier, allowing them to be the only alternative fuel source to glucose in brain [23]. These

molecules are transported via the blood throughout the body and are converted back into acetyl-CoA to enter the TCA cycle.

While animals cannot directly convert lipids into glucose like plants, glycerol produced through lipolysis of triglycerides can. Glycerol is converted to glyceraldehyde-3-phosphate, which directly enters the gluconeogenic pathway to ultimately produce glucose [24] (**Figure 1-2**). Glycerol has been shown to be a major gluconeogenic source in hibernating mammals, providing up to 75% of gluconeogenic substrate [25-27]. Additionally, in starving mice, glycerol is the largest carbon contributor for glucose formation [28]. This process allows for replenishment of glycogen stores in the liver and skeletal muscle, which are depleted during hibernation [27].

In order to facilitate the switch from glucose to fat as the main energy source, large changes in gene and protein expression must occur. In hibernating mammals, changes in gene expression reflects increased fatty acid metabolism capacity. The transcription factor PPARα appears to be a major regulator of this process. PPARα regulates the lipid metabolism that is activated during starvation, and is essential for ketogenesis [29]. PPARα is upregulated in mammalian hibernators, which correlates with activation of target genes [30, 31]. *PDK4*; a target gene of PPARα, is also upregulated during hibernation [32, 33]. PDK4 inactivates pyruvate dehydrogenase via phosphorylation, preventing metabolism of glucose by blocking conversion of pyruvate into acetyl-CoA [34].

In hibernating mammals, fatty acid binding proteins, which enable fatty acids metabolism and aids in their transport, are upregulated [33, 35-37]. Lipases, including *PNPLA2*, *HSL* and *PTL*, which hydrolyse triglycerides into glycerol and FFAs, are also upregulated in

various tissue-types during mammal hibernation [32, 33, 38]. Additionally, in brown adipose tissue, perilipins, which regulate access of lipases to triglyceride droplets, are also upregulated [33]. Both mitochondrial fatty acid metabolism genes and peroxisomal fatty acid genes are upregulated in the heart and skeletal muscle of hibernating ground squirrels [37]. This increased expression of fatty acid metabolic genes and glucogenic preventative genes appears to regulate the switch from glucose to lipid metabolism as the major fuel source.

1.5 Stress responses during hibernation

1.5.1 Hypoxia

Hibernating mammals reduce breathing and heart rates [39]. Consequently, oxygen availability to tissues and organs is reduced, causing some tissues to become hypoxic [40]. The ability of cells to withstand this lack of oxygen is essential to prevent otherwise lethal damage to vital organs. Unsurprisingly, hypoxia-associated enzymes are upregulated during hibernation. The hypoxia inducible factor subunit alpha (HIF-1α) is upregulated in brown adipose tissue, skeletal muscle and liver during hibernation, improving hypoxia tolerance [39, 41]. In normal oxygen conditions, HIF-1α is hydroxylated and targeted for degradation [42]. However, during hypoxia HIF-1α associates with HIF-1β, preventing hydroxylation and allowing stabilisation. The two subunits form the heterodimer HIF-1, which acts as a transcription factor to upregulate expression of key hibernation genes [42].

HIF-1 regulates two main functions during hypoxia: 1) the increased transport of oxygen to hypoxic tissue; and 2) the shift from aerobic respiration to anaerobic respiration in hypoxic cells [42]. In order to increase the delivery of oxygen to hypoxic tissue, HIF-1

stimulates the expression of vascular endothelial growth factor (VEGF). VEGF is a signaling protein that regulates angiogenesis and erythropoietin; a protein that regulates red blood cell production [43]. VEGF is upregulated in the hearts of hibernating woodchucks, where it is hypothesised to protect against myocardial ischemia by inducing angiogenesis or arteriogenesis [44].

1.5.2 Oxidative stress

A side effect of hypoxia during hibernation is oxidative stress during recovery from hypoxia after arousal [45]. It is hypothesized that the sudden increase in metabolic activity and oxygen consumption results in the increased production of reactive oxygen species (ROS). This occurs in marsh frogs (*Rana ridibunda*) after recovery from hibernation [46] and in Arctic ground squirrel (*Spermophilus parryii*) after arousal from hibernation [47]. To tolerate this, animals that undergo hypoxic conditions during torpor require antioxidant defenses and repair mechanisms [45]. An example of this in hibernating thirteen-lined ground squirrels is increased production of NRF2, a transcription factor that regulates the expression of antioxidant defense proteins [48]. SUD, AKR7A2, and HMOX1 are all antioxidant defense proteins that are upregulated by NRF2 [48]. In hibernating woodchuck (*Marmota monax*) heart catalase, responsible for catalysing hydrogen peroxide decomposition, is significantly upregulated during hibernation [49].

The upregulation of oxidative stress defenses during hibernation appears counterintuitive as oxidative stress predominantly occurs after arousal when the animal switches back to aerobic respiration. Two possible explanations exist: 1) early upregulation provides a prepared and rapid response to the upcoming oxidative stress; 2) they allow for increased protection of macromolecules from oxidative damage, even if low, during hibernation.

These explanations are not mutually exclusive, so a possible dual purpose for early upregulation remain possible [45, 50].

1.5.3 Apoptosis

The stressors experienced during hibernation are conducive to the induction of apoptosis (a form of programmed cell death). Apoptosis is characterized by several processes including cell shrinkage, nuclear DNA fragmentation, and membrane blebbing (bulging of the plasma membrane) [51]. Apoptosis is induced by two main pathways: 1) the extrinsic death receptor signaling pathway; and 2) the intrinsic mitochondrial pathway (Reviewed in [52]).

The extrinsic pathway is induced by the binding of death receptors to death receptor ligands [52]. Death receptor ligands are stimulated by extracellular signals such as growth factors, cytokines, and hormones. This results in activation of pro-caspase proteins into caspases, which are a family of proteases that result in caspase-3 activation and the progression of apoptosis [52]. In contrast, the intrinsic pathway is induced by internal stress signals, such as nutrient deprivation [53], hypoxia [54], high ROS concentration [55], or increased calcium concentration [55]. These encourage outer mitochondrial membrane permeabilization, causing the release of mitochondrial proteins such as cytochrome c (reviewed in [56]). Cytochrome c release allows for binding with Apaf-1 which forms the apoptosome complex. The apoptosome complex can recruit and activate caspase-9, which ultimately allows for activation of caspase-3, much like in the extrinsic pathway. In both cases, caspase-3 cleaves key cellular substrates causing the progression of many cellular events necessary for apoptosis [56].

Many of the intrinsic stress signals that results in apoptosis are experienced by hibernators [57]. Due to the devastating affects organism-wide induction of apoptosis can potentially have, hibernators have developed several mechanisms to prevent its progression [58]. In thirteen-lined ground squirrels, the caspase inhibitor x-IAP and anti-apoptotic Bcl proteins, which block mitochondrial permeabilisation, are induced during torpor [59, 60]. Additionally, anti-apoptotic protein kinases, such as Akt and GSK3β, are upregulated and activated during entrance and arousal from hibernation [59-61].

1.6 Tissue-specific responses to hibernation

1.6.1 Brain

As outlined in **Chapter 1.5.1**, induction of hibernation results in increased exposure to hypoxic conditions and an increased potential for oxidative stress damage. Furthermore, ischemia caused by a reduced heart rate also deprives cells from glucose. Hypoxic and ischemic insult is particularly pertinent to the brain as they can cause devastating effects on vital neural connections [62].

Deprivation of oxygen and glucose to neurons causes bioenergetic failure, resulting in a decreased ion potential across the plasma membrane [63]. Depolarization of the plasma membrane leads to increased synaptic release of glutamate, as well as the potential release of cytoplasmic glutamate into the extracellular space [63]. Increased extracellular glutamate results in excitotoxicity of neurons. Excitotoxicity is a pathological process that occurs when excitatory neurotransmitter glutamate receptors such as the *N*-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) are overactivated by excessive concentrations of glutamate [64-66]. Overactivation of glutamate receptors results in an influx of calcium ions (Ca^{2+}) into

the cell [67]. Excessive Ca^{2+} leads to activation of several pathways including caspase processing and ROS release that ultimately results in apoptosis (see **Chapter 1.5.2**). Reduced NMDAR function has been observed to occur in hibernating ground squirrels [68, 69]. Decreases in phosphorylation of the NMDAR NR1 subunit, a known activator of NMDAR function, and reduced Ca^{2+} influx via NMDAR was observed [68]. Furthermore, hibernating ground squirrels hippocampus possess increased tolerance to NMDA without induction of significant cell death compared to rat and interbout aroused squirrels [69].

Excitotoxicity is a well-known pathological condition associated with neurological disorders such as multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease [65]. The microtubule-associated protein tau, thought to be central to these disorders, forms pathological protein aggregations known as paired-helical-filaments (PHF) and neurofibrillary tangles (NFTs) [70]. Aggregations of tau are formed by the hyperphosphorylation of tau which causes its disassociation from microtubules [70]. Consequently, this causes the destabilization and polymerisation of microtubules. Much like in tauopathies, in hibernating mammals, tau is hyperphosphorylated [71-74]. However, rather than displaying pathological affects, this hyperphosphorylation is reversible and appears to be neuroprotective. This has led many to hypothesise that tau hyperphosphorylation in tauopathy may be a neuroprotective mechanism (as in hibernation) but when left unchecked it becomes pathological [70, 75, 76].

1.6.2 Heart

One of the most significant challenges hibernators must overcome is the maintenance of heart function and delivery of blood and nutrients to vital organs. Heart rate depression during hibernation is severe; with some animals reducing their heart rate by up to 95%; from 350-400 beats per minute (bpm) to less than 10 bpm [77]. Additionally, the low T_b experienced during hibernation causes increases in blood viscosity, making it even more challenging to pump blood. For example, decreases in blood temperature from 36.5°C to 22°C, which is much milder than that experienced by hibernators, causes a 26% increase in blood viscosity and, consequently, a 21% decrease in blood flow rate [78]. As such, hibernators require heart-specific adaptive strategies to increase the stroke volume and contractile strength of each individual heartbeat to overcome these problems.

One such strategy is the induction of reversible cardiac hypertrophy; which has been widely observed in both mammalian and reptilian hibernators [79-84]. Cardiac hypertrophy, also called ventricular hypertrophy, is a process that results in the thickening of the lower chamber (ventricle) of the heart [85, 86]. This process can be adaptive, known as physiological hypertrophy resulting from exercise and pregnancy, or maladaptive, known as pathological hypertrophy that can be caused by stress such as hypertension [85]. Physiological hypertrophy enhances cardiac performance by increasing the muscle mass and contractile force of the heart. Pathological hypertrophy may initially allow for increased heart function; however, prolonged stress ultimately leads to cardiac fibrosis and a reduced ability to pump blood. At the cellular level, cardiac hypertrophy involves the restructuring of cardiac muscle cells (cardiomyocytes) causing increased size, reorganisation of sarcomeres, and increased protein synthesis [86].

Hibernating cardiac hypertrophy appears to mirror physiological hypertrophy as it is quickly reversed upon arousal [87]. Transcription factors appear to govern the induction of cardiac hypertrophy by playing significant roles in cardiac remodelling [87-92]. The NFAT and MEF2 transcription factors are thought to regulate and promote increased protein synthesis in cardiomyocytes during hibernation. Furthermore, transcription factors, such as MyoG, appear to regulate expression of E3 ubiquitin ligases, such as MAFbx and MuRF1, in order to degrade cardiac proteins and restructure cardiomyocytes during mammalian hibernation [92].

1.6.3 Skeletal Muscle

Immobility and muscle disuse cause a decrease in muscle cell count, size, and contractile strength. In humans, muscle atrophy begins to occur after as little as five days of immobilisation [93]. During the hibernation season, animals undergo weeks or months of muscle inactivity. If atrophy and strength loss is left unchecked, this poses an enormous disadvantage to the animal as it reduces potential to exert physical activity post-arousal.

However, hibernators including bears, bats and squirrels, are (of varying degrees) resistant to skeletal muscle atrophy. In Greater tube-nosed bat (*Murina leucogaster*), no signs of atrophy or muscle tension reduction is observed over the three month hibernation season [94]. Hibernating skeletal muscle is associated with shifts from fast myosin isoforms to slow isoforms, suggesting a general switch to slow-twitch muscle fibres [95]. This switch is thought to be mediated by the activation of the PCG-1α endurance exercise pathway and upregulation of the muscle-specific transcription factor MEF2 [89, 96]. Furthermore, essential metabolic and sarcomeric protein levels that are necessary for muscle contraction are maintained with actin and voltage channel proteins being

upregulated [94]. Despite the global reduction in gene expression rates, the induction of protein biosynthesis during the regular arousal periods may counteract the atrophy caused by muscle disuse [97]. This is supported by the upregulation of genes associated with protein biosynthesis and ribosome biogenesis during arousal [97].

Regulation of proteolytic and autophagic pathways are also thought to contribute to the prevention of muscle atrophy during hibernation [98]. Pro-proteolytic transcription factors such as FOXO1 are downregulated during hibernation [95], while protease inhibitors such as Calpastatin are upregulated [99]. SGK1, a mediator of skeletal muscle homeostasis, has been identified as a key gene in prevention of muscle atrophy during hibernation [98]. When SGK1 is overexpressed in mice, they are protected against disuse atrophy and starvation-induced muscle atrophy; suggesting an important role not just in hibernators [98]. Furthermore, the anti-proteolytic affects experienced by hibernating mammals may also be mediated by proteins released into the blood. When muscle cells of rats are incubated with plasma from hibernating bears, there was a 40% decrease in the net proteolytic rate [100]. This was further complemented by downregulation of the lysosomal and ubiquitin proteolytic pathways.

1.7 Regulation of the hibernation phenotype

For animals to initiate, maintain, and control the large changes in cellular physiology associated with hibernation, many regulatory mechanisms must be employed. The regulation of cellular processes involved in hibernation has been extensively explored in mammals. Regulation from transcription to post-translation have been observed to control vital aspects of the hibernation phenotype. Gene expression regulation is controlled by epigenetic mechanisms including chromatin modification, DNA methylation, and

miRNA-mediated gene silencing [14, 101-106]. Control and preservation of protein function is mediated by mechanisms such as protein ubiquitination, SUMOylation, and phosphorylation [5, 7, 107-111].

1.7.1 DNA methylation

DNA methylation is a core epigenetic transcriptional regulatory mechanism in vertebrates [112]. It is involved in key biological processes, including cellular differentiation, tissue-specific gene expression, aging, cancer, and X-chromosome inactivation. DNA methylation involves the covalent addition of methyl groups (CH_3) to cytosine, and in some cases, adenosine bases. Cytosine methylation (5-methylcytosine – 5mC) can exist in three contexts; CpG, CHG and CHH (where H is A, T or C). However, CpG methylation is the predominant form in vertebrates (reviewed in [113]). The prevailing hypothesis for the function of CpG methylation is that it: 1) acts to physically modulate the access of transcriptional machinery and transcription factors to the DNA [114]; and 2) binds methyl-CpG-binding domain (MBD) proteins which modify the histone structure by recruiting histone modifying proteins [115]. Hereinafter, DNA methylation will refer to CpG methylation alone.

DNA methylation is mediated by DNA methyltransferases (DNMTs) that can either maintain methylation status after replication (maintenance methylation) or deposit new methylation marks (*de novo* methylation) [116]. Demethylation of DNA is mediated by either passive loss or active removal [116]. Passive loss of 5mC occurs when the maintenance DMNT (DNMT1) fails to methylate the newly synthesised DNA strand, ultimately resulting in dilution of 5mC in the descendant cell population. The mechanism for active demethylation in animals remains more obscure. Currently it is thought that

active demethylation is mediated by the conversion of 5mC into oxidised derivatives by ten-eleven translocation (TET) family of proteins [112, 116]. 5mC is firstly converted into 5-hydroxymethylcytosine (5hmC), which is subsequently converted into 5-formylcytosine (5fC) and, finally, into 5-carboxycytosine (5caC). Both 5fC and 5caC can be excised by thymine DNA glycosylase (TDG), allowing for unmodified cytosine to be reintroduced by the base excision repair (BER) pathway.

In somatic human cells, ~70% of CpG dinucleotides are methylated, with a concentration around gene promoters. CpG sites are non-randomly distributed across the genome of vertebrates, clustering in regions called CpG islands (CGIs). CGIs are regulatory features that contain high GC content (> 50%) and are of considerable size (>200 bp) [117]. These features are usually localised to gene promoters and, to a lesser degree, gene bodies. CGIs are mostly lowly methylated, with CGIs particularly in the promoter region and the first exon associated with gene repression [118]. Furthermore, the dynamic methylation up- and downstream of CGIs (i.e. CGI shores and shelves) is also responsible for the modulation of gene expression [119].

Research into the role of DNA methylation in hibernation is relatively sparse with only three studies, all of which are in small mammals [103, 104, 120]. In hibernating mammals, global DNA methylation dynamics is tissue specific. In brown adipose tissue, global DNA methylation was 1.7-fold higher during hibernation than euthermia [103], while in skeletal muscle total DNA methylation was ~25% lower during late torpor [104]. Furthermore, mRNA expression of DNMTs and protein expression of MBD1 are dynamic and tissue specific during hibernation [103, 104]. However, these studies only focused on total DNA methylation, rather than CpG methylation. Therefore, whether these changes in total DNA methylation correlate with changes in transcription is

unknown. The CpG methylation of a hibernation-specific gene in chipmunk (*HP-27*) was assessed [120]. In liver, a CpG dinucleotide in the promoter region that corresponded to a transcription factor binding site was hypomethylated, correlating with downregulation of the gene. These observations indicate that changes in DNA methylation appear to have a role in regulating responses during hibernation. However, more research is needed to elucidate specific genes and pathways under control of differential CpG methylation.

1.7.2 Histone modifications

Eukaryotic DNA is packaged into nucleosomes, that is DNA wrapped around dimers of the core histone proteins, H2A, H2B, H3 and H4 (reviewed in [121]). Nucleosomes form the basic unit of chromatin and are interconnected by sections of linker DNA and associated linker histone proteins (H1 and H5). Histone proteins can bind adjacent nucleosomes through a positively charged tail. Post-translational modifications to amino acid residues on these tails allows for modulation of chromatin structure. An example of a common modification is trimethylation of the amino acid lysine (K) 27 on histone 3 (H3), known as H3K27me3. Modifications can cause either compaction or loosening of the chromatin structure.

Chromatin exists in two basic forms: 1) heterochromatin; and 2) euchromatin [121]. Heterochromatin is transcriptionally silent chromatin. It is very tightly packed and bound to heterochromatic proteins that function to maintain the tight structure and prevent transcription of the DNA [122]. In contrast, euchromatin is transcriptionally active. It is distinguished by its loose packaging and association with transcription factors and RNA polymerase II [121]. Heterochromatin has modifications that are correlated with transcriptional regression (e.g. di- and trimethylated H3K9 and H3K27) whereas

euchromatin has modifications associated with transcriptionally active genes (e.g. acetylated H3K9 and H3K27, and monomethylated H3K4, H3K9 and H3K27 [123]. DNA methylation is strongly associated with histone modifications with methyl binding proteins recruiting histone modifying proteins [124].

The activity and expression of histone modifiers, and the consequent modification of histones themselves is globally altered during the hibernation season [101-104, 125, 126]. Specifically, the roles of both lysine acetylation and methylation; two key modifications for transcriptional activation and repression, respectively, have been explored. This modulation in histone dynamics is reversible and fluctuates over torpor and arousal periods [125, 126]. The histone deacetylases HDAC1 and HDAC4 have ~1.5-fold higher expression during hibernation than euthermia in brown adipose tissue and skeletal muscle of ground squirrel [101, 103]. In turn, HDAC activity was found to be 1.82-fold higher during hibernation compared to euthermia. HDACs are typically considered negative regulators of transcription as the removal of acetyl groups increases the positive charge of histone tails, thereby condensing the chromatin structure and preventing transcription [127]. This consequently leads to reductions in histone H3 modifications associated with active transcription, including a 25-50% decrease in H3K23 acetylation [101].

Histone modifications associated with transcription (i.e. acetylation of H3K14, H3K18 and H3K27) were increased during early arousal from hibernation in ground squirrel skeletal muscle [125]. Three histone lysine methyltransferases, SMYD2, SET7 and SET9, were differentially expressed during ground squirrel hibernation [126]. During torpor, SMYD2 was downregulated whereas SET7 and SET8 were upregulated. All three of these proteins monomethylate (me1) H3K4, and SMYD2 also monomethylates H3K36. Consequently, increased levels of H3K4me1 was observed during torpor. Furthermore,

H3K9me3 (a known repressive mark) was also increased during torpor. Collectively, the current research suggests that hibernation results in global modulation of both active and repressive histone marks. Therefore, targeted experimentation, such as chromatin immunoprecipitation sequencing (ChIP-seq), is necessary to elucidate the specific genes under control of these modifications.

1.7.3 microRNAs

In recent years, the role of miRNAs in the post-transcriptional regulation of mRNAs has become increasingly evident due to advances in small RNA sequencing. miRNAs are short, 17 to 22 nucleotide RNA molecules that have many regulatory functions including the degradation and storage of mRNA molecules, and repression of translation [128]. miRNAs are transcribed as longer molecules with unique secondary hairpin structures known as primary miRNAs (pri-miRNAs). Pri-miRNAs are processed in the nucleus by the RNase Drosha into precursor miRNAs (pre-miRNAs) that are transported into the cytoplasm. Pre-miRNAs are further processed by the RNase Dicer into functional mature miRNAs (reviewed in [129]).

In humans, miRNAs are known to target over 60% of all protein-coding genes [130]. Due to the very low substitution rate of mature miRNA sequences (at 3.5%), mature miRNAs are well conserved between Metazoans [131, 132]. miRNAs target mRNA sequences through the 5’ seed region (nt 2 -8) which complement the 3’-untranslated region (UTR) of mRNAs [133]. This allows for the RNA-induced silencing complex (RISC) to be recruited, which cleaves and degrades the mRNA transcript [129]. More recently, RISC has been shown to repress the translation of mRNAs without degradation by modulating the binding of ribosomes and ribosome-associated proteins [134].

Due to the role of miRNA-mediated regulation in many biological processes such as cellular differentiation, metabolism, apoptosis, and cancer [133], the role of miRNAs in regulating hibernation has been studied extensively [105, 106, 135-141]. The first study to investigate the role of miRNAs in hibernators discovered upregulation of miR-1 and miR-21 during torpor [140]. miR-1 is associated with the regulation of cell proliferation, whereas mir-21 is anti-apoptotic. Both are important during hibernation. Using RNA-seq changes in miRNA expression have been shown to correlate with several critical responses associated with hibernation. miRNAs appear to regulate important shifts in metabolism including metabolic depression and switches in cellular fuel sources in the liver, brain, and skeletal muscle of hibernating mammals [105, 106, 135]. Tissue-specific responses also appear to be mediated by miRNA action. In skeletal muscle, miRNAs important in muscle maintenance and prevention of atrophy are increased during hibernation [105, 135, 139]. Increased neuroprotection in brain is also regulated by the action of miRNAs [137].

1.7.4 Protein phosphorylation

The existing literature states that one of the main mechanisms that allows for the reduction in cellular activity is reversible protein phosphorylation [110, 111]. Protein phosphorylation is a post-translational modification whereby a phosphate group is covalently bound to specific amino acids. Catalysis of protein phosphorylation is mediated by protein kinases, while catalysis of dephosphorylation is mediated by protein phosphatases [5]. The addition of a phosphate group alters the structural confirmation of proteins, allowing for changes in function, such as enzymatic inactivation.

Reversible protein phosphorylation-mediated regulation is thought to govern key metabolic processes in mammalian hibernation [5]. In skeletal muscle of the hibernating ground squirrels, the depression of cellular ion transport of Na+/K+-ATPase is thought to be guided by reversible phosphorylation [11]. Phosphorylation of Na+/K+-ATPase may cause inactivation of enzyme function, which plays a key role in the depression of the basal metabolic rate of the organism [11]. The depression of protein translation during hibernation has also been attributed to phosphorylation of proteins associated with protein biosynthesis [9, 142].

Reversible phosphorylation of metabolic enzymes and other proteins could allow for the shutdown of specific cellular processes that are not necessary during the torpid state. In a more general sense, this regulatory mechanism has been shown to arrest and depress the global synthesis of proteins [9]. Ribosomal initiation and elongation factors are phosphorylated during torpor, potentially causing enzymatic inactivation and the prevention or reduction in the capability of cells to initiate and continue translation [9, 142]. Protein phosphorylation may also be a leading mechanism in the regulation of metabolic depression in anoxia tolerant reptiles, which is an adaptive response similar to hibernation [143]. During a hypoxia state in turtles, protein phosphorylation is associated with the control of cellular functions including glycolytic enzymes [144], voltage-gated ion channels [145], and protein synthesis [110].

1.7.5 Small ubiquitin-like modifying proteins

Small ubiquitin-like modifier (SUMO) proteins are ~100 amino acids in length and resemble ubiquitin. The covalent bonding of SUMO proteins (SUMOylation) is a post-translational modification involved in important biological processes, such as apoptosis,

transcriptional regulation, and stress response [146]. A major target for SUMOylation are transcription factors, typically resulting in significant decreases in gene expression [147]. SUMOylation of transcription factors causes alterations in DNA binding affinity, subcellular localisation, interactions with co-regulators, and chromatin structure [148].

Protein SUMOylation is notably important in stress response, mediating activity of proteins such as p53 in response to cerebral ischemia and oxidative stress [149-152]. As prevention of ischemia and oxidative stress damage is extremely pertinent to hibernators (see **Chapter 1.5.1**, **1.5.2** and **1.6.1**), it is unsurprising that SUMOylation has a role in regulation during hibernation [3]. During ground squirrel hibernation, levels of SUMO-conjugated proteins are increased in the brain, liver, and kidney. This correlates with increased expression of UBC9, a SUMO-conjugating enzyme [109]. Immunohistochemistry revealed localisation of SUMO-1 protein in neuronal bodies is restricted to hibernating squirrels. Together with the fact that overexpression of UBC9 or SUMO-1 in neurons protects against ischemic damage, SUMOylation potentially serves as a critical regulator of neuroprotection against glucose and oxygen deprivation during hibernation [153].

1.8 The Australian central bearded dragon

Hibernation traditionally refers to the state endotherms enter during winter months to withstand the cold temperatures and lack of food. However, hibernation also occurs in many ectotherms, such as in the reptile the Australian central bearded dragon [2]. Current hibernation research is mainly focused on mammals, such as bears and squirrels [97, 100, 154, 155]. Research into the molecular mechanisms of hibernating reptiles has been very limited. Many refute the use of the word ‘hibernation’ to refer to periods of winter

dormancy in reptiles due to the lack of active body temperature regulation and suggest the use of the word 'brumation' instead [156]. While reptilian hibernation differs from the "true" hibernation utilised by mammals such as ground squirrels, recent evidence has begun to suggest that the physiology of mammalian hibernation is highly variable between species.

Core differences even exist between two classic models of hibernation: the thirteen-lined ground squirrel and brown bears (*Ursus arctos*). Ground squirrels exhibits decreases in T_b to as low as -2.9°C [157], while brown bears exhibit only mild reductions to ~33°C [158]. One of the most extreme examples of variation within mammalian hibernation is exemplified by common tenrecs (*Tenrec ecaudatus*) [159]. Unlike any other mammalian hibernators, T_b does not determine whether metabolic depression occurs in tenrecs, as they can remain active at 12°C while hibernate at 28°C [159]. Rather T_b follows that of environmental temperatures where they are buried ~1 metre under soil, like hibernating reptiles. Due to this, tenrecs lack IBAs during hibernation like reptiles [160]. These variations suggest that hibernation is not one specific physiological response, and as such, herein 'hibernation' will be used to refer to the state of reptilian winter dormancy.

The Australian central bearded dragon is an agamid lizard that is native to the arid and semiarid regions of Central Australia. In the wild, bearded dragons hibernate during the coldest months of the year (between May and September), where ambient temperatures range from 5°C to 18°C [2]. During this period bearded dragons seek refuge, burying themselves in the soil or in fallen logs and tree stumps. While the physiological responses during bearded dragon hibernation are unknown, the nature of the hibernaculum (buried) suggests reduced respiratory and heart rates must occur. As with tenrecs, T_b reflects that

of the environment [2, 159]. Additionally, bearded dragons are only known to rewarm through basking.

Similar to hibernating mammals, hibernating reptiles store large amounts of lipids prior to hibernation and utilize it as the main energy source, particularly in the liver and fat bodies in the tail (reviewed in [161]). Stored lipids appear to be metabolised through β-oxidation and converted into the ketone β-hydroxybutyrate. Additionally, stored glycogen appears to be a secondary source of energy during reptilian hibernation [162]. In hibernating reptiles, the oxidative metabolic rate is slightly less depressed than in hibernating mammals, which may be due to the increased reliance on oxidative metabolism of stored glycogen [163, 164].

Recently, the central bearded dragon is of increased biological interest due to its ability sex reverse genetically male individuals to a female phenotype through high incubation temperatures of eggs [165]. This has led to a wealth of molecular studies on this species and the completion of its genome sequence [165-169]. For these reasons, the central bearded dragon is an excellent model to study reptilian hibernation.

1.9 Use of omics technologies

Omics technologies aim to characterise and quantify the collective molecular makeup responsible for the structure, form and function of specific cell and tissue types [170]. These technologies allow for the study of core biological systems including; genes (genomics), DNA methylation (methylomics), RNAs (transcriptomics), proteins (proteomics), and metabolites (metabolomics). Omics technologies allow for a top-down experimental approach. Broad questions can be addressed to generate more specific

hypotheses. An advantage of omics technologies is the ability to correlate biomolecule abundances across multiple technologies (multiomics). This is particularly useful for investigating non-steady state conditions such as hibernation where molecular processes may not be functioning as they do in steady state and further validation is necessary [171]. As many RNA species exist within sub-cellular locations such as P-bodies or stress granules, whole tissue omics may not reflect the true abundance of free or active RNA molecules. However, employing these technologies provides clear advantages to traditional methods, such as qPCR, as it allows for a system-wide understanding of physiological processes, which is invaluable to the field of hibernation.

1.9.1 Transcriptomics

The study of an organism's whole transcriptome was not possible until the advent of high-throughput sequencing technologies. Previously, transcriptomes could only be partially characterised and quantified by techniques such as RT-qPCR and microarrays. Microarrays permit a measurement of the abundances of known transcript through hybridisation of thousands of probes [172]. In contrast, RNA sequencing (RNA-seq) allows for the direct sequencing of both known and unknown transcripts in any model species. RNA-seq has high sensitivity, requires low quantities of input RNA, and can detect splice variants and single nucleotide polymorphisms (SNPs). Perhaps most importantly, RNA-seq does not require a reference genome or transcriptome [172]. For these reasons, RNA-seq is the gold standard for studying transcriptomes.

RNA-seq is now extensively adopted to study the transcriptomes (mRNA) of animals throughout hibernation, including: brain, heart, skeletal muscle, brown and white adipose tissue of ground squirrels [33, 108, 155, 173]; white adipose tissue of dwarf lemurs [174,

175]; liver, adipose tissue, skeletal muscle of grizzly bears [176]; kidneys of brown bears [177]; and wings of little brown bats [178]. However, RNA-seq has only been used once to study miRNA dynamics in hibernating little brown bats [105], with most studies focusing on known miRNAs using RT-qPCR technologies [106, 135-137, 139, 140]. RNA-seq allows the study of thousands of miRNA species, including discovery of novel miRNAs that may be crucial in regulating the hibernation phenotype. As such, the continued use of RNA-seq to study the transcriptome of hibernators will undoubtedly provide invaluable knowledge about the mRNA, non-coding RNA dynamics throughout hibernation.

1.9.2 Proteomics

High-throughput proteomic technologies allow for the simultaneous identification and quantification of potentially thousands of proteins in a sample. Traditionally, antibody-based technologies such as ELISA and western blots were used to study the proteome. These only permit quantification of a small number of known proteins. The current gold standard for high-throughput proteomics is mass spectrometry (MS) [170]. Biomolecules, such as proteins, are ionized and separated by their mass-to-charge ratio (m/z), creating a mass spectrum that allows for identification.

Depending on the technology used, MS allows both relative and absolute quantification of proteins. There are two main approaches for quantification: 1) stable isotope labelling, the most commonly used being isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT); and 2) label-free quantification, with spectral counting being the most commonly used method. Labelling approaches are generally

more accurate, but label-free strategies are less expensive [179]. However, both approaches can provide important information to address specific biological questions.

Proteomic technologies have been extensively used to study protein dynamics in hibernating mammals, including Daurian squirrels, thirteen-lined squirrels, arctic ground squirrels and greater mouse-eared bats [180-185]. These studies have revealed proteins essential to hibernation that would otherwise remain unknown [186]. Furthermore, proteomic analyses have revealed that protein changes between torpor and euthermic mammals are typically low compared to mRNA changes, less than two-fold different (reviewed in [186]). Accordingly, many changes in protein function are hypothesised to be mediated by post-translational modifications, which can be identified by MS. The multiomic approach of comparing transcriptomics and proteomics was successfully utilised to study the skeletal muscle of hibernating ground squirrel [173]. Therefore, the use of proteomic analyses is essential to understand the changes in protein abundances and modifications that make hibernation possible.

1.9.3 Methylomics

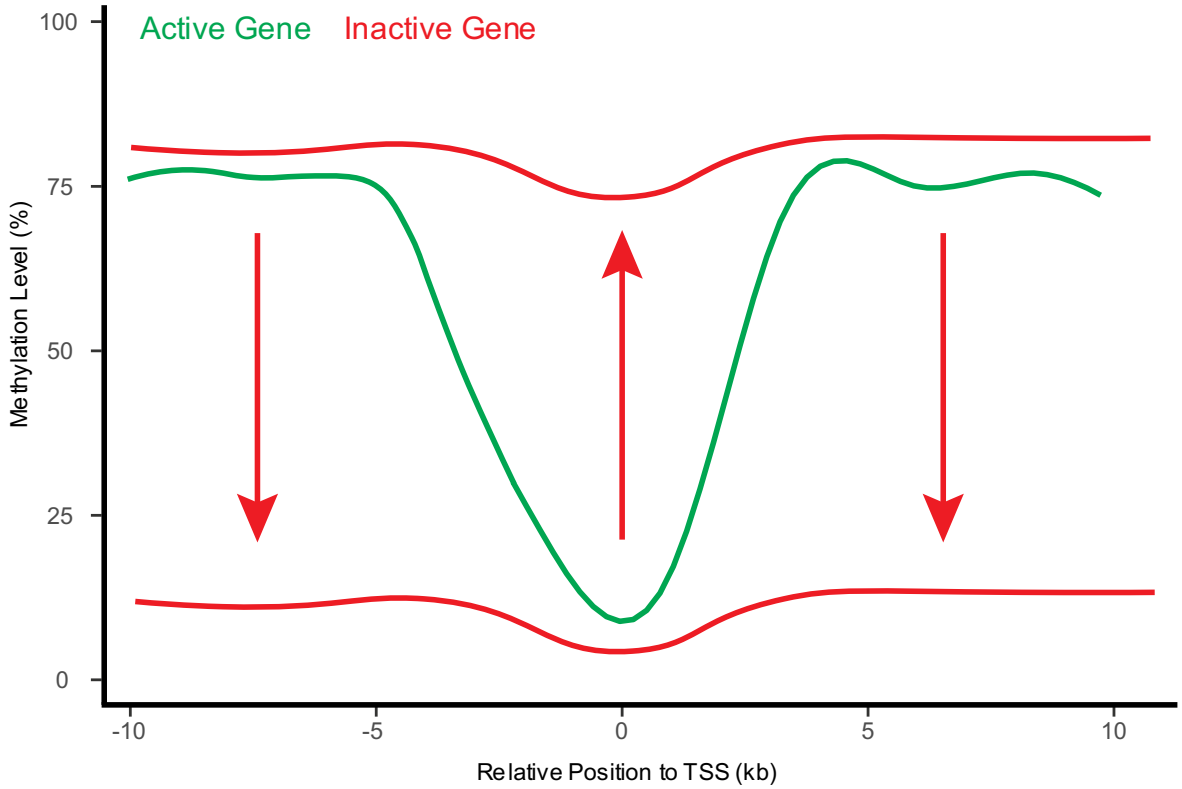
High-throughput bisulfite sequencing (BS-seq) permits single nucleotide resolution of DNA methylation [187]. DNA is bisulfite treated, which converts unmethylated cytosines to uracil, whereas methylated cytosines (5mC) remain unaffected. This is followed by standard DNA sequencing. Methylated cytosine residues remain as cytosine upon sequencing, but unmethylated cytosines are sequenced as thymine. There are two main forms of BS-seq: 1) whole genome bisulfite sequencing (WGBS); and 2) reduced representation bisulfite sequencing (RRBS) [187].

WGBS is considered the gold standard for studying DNA methylation as it provides genome wide sampling. The DNA is simply bisulfite converted and then sequenced. In contrast, RRBS requires digestion of genomic DNA with restriction enzymes, such as MspI, which preferentially target regions of high CpG density. This enriches sequencing libraries for CpG-dense regions (i.e. CGIs), which are thought to be important in gene regulation [187]. As with other omics, methylomics can be utilised in multiomics to examine a given biological question. Specifically, correlating with transcriptomics is vital to understanding how change in CpG methylation may result in change in mRNA expression.

A major issue with bisulfite sequencing is that it cannot distinguish between 5mC and 5hmC. Both modified bases get read as cytosine upon sequencing [188]. 5mC and 5hmC have inherit differences in function, therefore, treating both as the same base in BS-seq data could lead to incorrect functional interpretation [188]. However, recent advances have led to the development of technologies that can distinguish 5mC from 5hmC, as well as the other 5mC deamination derivates (see **Chapter 1.7.1**). Oxidative bisulfite sequencing (oxBS-seq) distinguishes between 5mC and 5hmC [188]. Chemical modification-assisted bisulfite sequencing (CAB-seq) identifies 5caC [189], whereas methylase-assisted bisulfite sequencing (MAB-seq) detects of both 5fC and 5caC [190]. Collectively, these four approaches detect, at single nucleotide resolution, 5mC and its oxidated intermediates. This could reveal genomic regions that are actively being demethylated, versus passively demethylated. Active versus passive DNA demethylation has never been explored in hibernation.

Across different tissues and between species, RRBS and WGBS has led to genome-wide knowledge of the role CpG methylation plays in gene regulation. The use of BS-seq has

reinforced the importance of transcription start sites (TSS) of genes for DNA methylation-mediated regulation [191]. Specifically, 1 kb up- and downstream of TSSs show the greatest correlation with gene expression. This was corroborated by the observation that



increased methylation (hypermethylation) of first exon of a gene correlates with increased gene repression. BS-seq supports an emerging idea that rather than absolute methylation in promoter regions, the spatial distribution of methylation across the TSS correlates with silencing or activation of gene expression (**Figure 1-3**) [192-194]. Both universally high, and universally low levels of DNA methylation correlate with low gene expression. In contrast, low DNA methylation at the promotor that is flanked by high DNA methylation correlates with increased gene expression.

Figure 1-3. Generalised DNA methylation profile across transcription start sites of active (green) and inactive (red) genes.

To date, BS-seq has not been used to examine hibernation in any system, with only assay-based analyses employed (see **Chapter 1.8.1**) [103, 104]. BS-seq technologies will unquestionably provide critical information about CpG methylation dynamics during the hibernation season.

1.10 Biomedical applications of hibernation research

The exceptional ability of hibernators to withstand physiological stress that would be life threatening to many species, including humans, makes them a unique source of information for several biomedical applications. By understanding the strategies and mechanistic controls that allow hibernators to survive adverse conditions, improved treatments for diseases could be discovered, such as: ischemia-reperfusion, stroke, cardiac arrest, metabolic diseases, tauopathies, and others.

Targeted temperature management (TMM) or therapeutic hyperthermia is the controlled decrease in body temperature to 33°C or 36°C [195]. TMM has been increasingly used to treat conditions that cause reduced blood flow, such as cardiac arrest or stroke. It effectively increases survival and neurological outcomes following cardiac arrest in humans [196] and aids in neurological protection following strokes in animal models [197]. However, TMM has potential complications, including infection, bleeding, and high blood sugar [195]. Insights into hibernation could lead to improved therapeutic hyperthermia by reducing the number of complications, and allowing for lower body temperatures, which usually result in tissue damage and death [198]. Additionally, a better understanding could be used to improve the survival of organs and tissues in transplantations. The current technique to preserve organs for transplantation is cooling in ice-water solutions. Although effective, organs can only tolerate short exposure times

(from six hours to 30 hours depending on the organ) as damage caused by oxidative stress and inflammation quickly occur [199]. Understanding how hibernators deal with these stressors could provide clues about how to reduce tissue damage, thereby improving the quality and shelf-life of organ and tissue transplants.

Hibernators, including the central bearded dragon [2], possess a remarkable ability to prevent skeletal muscle atrophy during hibernation. Humans, in contrast, experience severe atrophy during periods of muscle disuse and immobilisation [200]. Further investigation of the molecular mechanisms that reduce muscle atrophy in hibernating animals may yield human applications, such as reducing muscle atrophy caused by muscle disuse or the microgravity experienced by astronauts [200, 201].

The remodelling of metabolism that occurs during hibernation could also guide understanding of human metabolic syndrome (e.g. obesity and diabetes) [202]. During hibernation, hibernators are typically obese and develop natural insulin resistance, analogous to human obesity and diabetes. However, this is quickly reversed after arousal with no negative side-effects associated with human obesity and diabetes [202, 203]. Investigation of hibernation in a variety of model systems could inform new avenues of research with exciting applications.

1.11 Summary

The ability of animals to enter a state of hypometabolism during hibernation is attributed to the maintenance and regulation of very specific metabolic pathways. Reprioritisation of energy consumption, and the downregulation of unnecessary functions, is necessary to ensure the viability of animals during hibernation. Additionally, the upregulation of processes such as lipid metabolism, hypoxia tolerance, oxidative-stress defence, neuroprotection and prevention of muscle atrophy, are vital in ensuring survival – not only during torpor but also after arousal. The regulation of these processes is mediated by many mechanisms, including reversible protein phosphorylation, SUMOylation, miRNAs, DNA methylation, and histone modifications. The use and coordination of omics technologies such as RNA-seq, quantitative proteomic MS and BS-seq is essential for studying this complex phenotype. Current research in the molecular biology of hibernation predominantly focuses on mammalian systems. However, research of reptilian systems such as *Pogona vitticeps* will provide evolutionary context and crucial insight for understanding this fascinating process.

2 Gene expression changes during hibernation

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2.1 Gene expression profiling reveals adaptive strategies of the hibernating reptile *Pogona vitticeps*.

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I performed all the experiments and most analyses for this manuscript. These include RNA extraction, protein extraction, and bioinformatics and statistical analysis of RNA-seq and proteomics data.

Your contribution to writing the paper:

I wrote all manuscript drafts and generated all figures.

Comments:

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2.2 Published manuscript

Waking the sleeping dragon: gene expression profiling reveals adaptive strategies of the hibernating reptile *Pogona vitticeps*.

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Key words: Hibernation, *Pogona vitticeps*, central bearded dragon, RNA sequencing, proteomics, miRNA, epigenetics, stress response

2.2.1 Abstract

Background: Hibernation is a physiological state exploited by many animals exposed to prolonged adverse environmental conditions associated with winter. Large changes in metabolism and cellular function occur, with many stress response pathways modulated to tolerate physiological challenges that might otherwise be lethal. Many studies have sought to elucidate the molecular mechanisms of mammalian hibernation, but detailed analyses are lacking in reptiles. Here we examine gene expression in the Australian central bearded dragon (*Pogona vitticeps*) using mRNA-seq and label-free quantitative mass spectrometry in matched brain, heart and skeletal muscle samples from animals at late hibernation, two days post-arousal and two months post-arousal.

Results: We identified differentially expressed genes in all tissues between hibernation and post-arousal time points; with 4264 differentially expressed genes in brain, 5340 differentially expressed genes in heart, and 5587 differentially expressed genes in skeletal muscle. Furthermore, we identified 2482 differentially expressed genes across all tissues. Proteomic analysis identified 743 proteins (58 differentially expressed) in brain, 535 (57 differentially expressed) in heart, and 337 (36 differentially expressed) in skeletal muscle. Tissue-specific analyses revealed enrichment of protective mechanisms in all tissues, including neuroprotective pathways in brain, cardiac hypertrophic processes in heart, and atrophy protective pathways in skeletal muscle. In all tissues stress response pathways were induced during hibernation, as well as evidence for gene expression regulation at transcription, translation and post-translation.

Conclusions: These results reveal critical stress response pathways and protective mechanisms that allow for maintenance of both tissue-specific function, and survival

during hibernation in the central bearded dragon. Furthermore, we provide evidence for multiple levels of gene expression regulation during hibernation, particularly enrichment of miRNA-mediated translational repression machinery; a process that would allow for rapid and energy efficient reactivation of translation from mature mRNA molecules at arousal. This study is the first molecular investigation of its kind in a hibernating reptile, and identifies strategies not yet observed in other hibernators to cope stress associated with this remarkable state of metabolic depression.

2.2.2 Background

Hibernation is an extreme state of inactivity used among diverse animal lineages to cope with low or unpredictable food availability and unfavourable seasonal conditions during winter. Hibernation involves long periods of hypometabolism (torpor), often interrupted by shorter periods of euthermia (interbout arousal). These periods of euthermia allow animals to rewarm and replenish gene and protein products; processes that are virtually halted during torpor [204]. Two decades of molecular studies of hibernation have focused on mammals, such as bears and squirrels [7, 92, 97, 100, 106, 155, 205], and recently marsupials [206], with little consideration of hibernation in reptiles. Debate surrounds the use of the word 'hibernation' in reptiles, with the thought that the lack of active body temperature regulation and inconsistent use of torpor necessitates an alternative term, i.e. 'brumation' [156]. However, there is large variation in physiology even between hibernating mammals, notably in tenrec [159], implying that hibernation is not one specific physiological state. As such, herein 'hibernation' will be used to describe the state of reptilian winter dormancy. Much like in mammals, reptilian hibernation also involves radical changes in behaviour and physiology [2].

In mammals, hibernation is achieved through a complex reprogramming of biological processes that leads to a drastic reduction in basal metabolic rate, transcription and translation, oxygen consumption, heart rate, and core body temperature, and an increase in physiological stress tolerance [5]. Hibernators employ general adaptive responses across all tissue types and exhibit a range of tissue-specific responses. For example, during hibernation neuroprotective processes are activated in the brain (reviewed in [8]), contractive strength is increased in the heart [80, 207], and atrophy is limited in skeletal muscle [92, 207].

Hibernation in mammals is governed transcriptionally via chromatin modification and DNA methylation, post-transcriptionally via microRNAs (miRNAs), and post-translationally via protein modifications such as SUMOylation (reviewed in [5]). While non-cleavage translational repression of mRNAs via miRNAs is thought to be important in mammalian hibernation, as of yet, there is no direct evidence.

Exploring mechanisms used by different species to cope with extreme conditions and stressors may yield information pertinent to human disease, such as age-associated neurodegeneration, muscle atrophy, and ischemia-reperfusion injury. Studies on non-traditional model species, which have evolved different physiological strategies to cope with extreme and variable conditions, provide this critically important perspective. The Australian central bearded dragon (*Pogona vitticeps*) is an excellent model to study reptilian hibernation because the genome is sequenced [166], and hibernation that mimics natural hibernation can be easily induced in captivity. Under natural conditions, bearded dragons hibernate by burying themselves in the soil or seeking refuge in fallen logs or tree stumps [2]. Typically, hibernation occurs between May and September, the coldest months of the year, where temperatures range from 5°C to 18°C. While physiological studies of bearded dragon hibernation in the wild is lacking, the nature of hibernation sites (buried) suggests the lizards have reduced breathing and heart rates during hibernation, with body temperatures reflecting that of ambient temperature (as they are ectothermic). However, unlike mammalian hibernators, the central bearded dragon is not known to have interbout arousals, with rewarming achieved through basking after arousal.

We profiled gene expression using mRNA sequencing (mRNA-seq) in three tissues (brain, heart and skeletal muscle) at three time points: 1) late hibernation; 2) two days post-arousal (pre-feed); and 3) two months post-arousal. We performed label-free

proteomic quantification in the same three tissues at two time points: 1) late hibernation and 2) two months post-arousal. Differentially expressed genes and proteins were analysed to determine overrepresented biological pathways during hibernating and waking periods. We discovered tissue-specific pathways that protect against the stress of reptilian hibernation and provide the evidence for multiple levels of gene expression regulation that may govern the physiological changes associated with hibernation.

2.2.3 Results

2.2.3.1 Differential Gene and Protein Expression

Hierarchical clustering of the 3,000 most highly expressed genes discovered with RNA-seq in brain, heart, and skeletal muscle grouped samples of the same tissue (**Figure 2-1A**). Within the tissue-specific clusters, three biological replicates of hibernating individuals were separated from the two post-arousal time points, which clustered together as a single group. Since the two post-arousal time points clustered together and differential gene expression analysis revealed minor differences in expression between the two post-arousal time points (**S2-1 Table**), they were treated as a single time point for all subsequent differential gene expression analyses. The greatest number of differentially expressed genes between hibernating and aroused animals was observed in skeletal muscle, followed by heart, and then brain (**Figure 2-1B, S2-1 Table**). A subset of 1311 genes was upregulated in all tissue types during hibernation and 1171 genes were downregulated (**Figure 2-1B**).

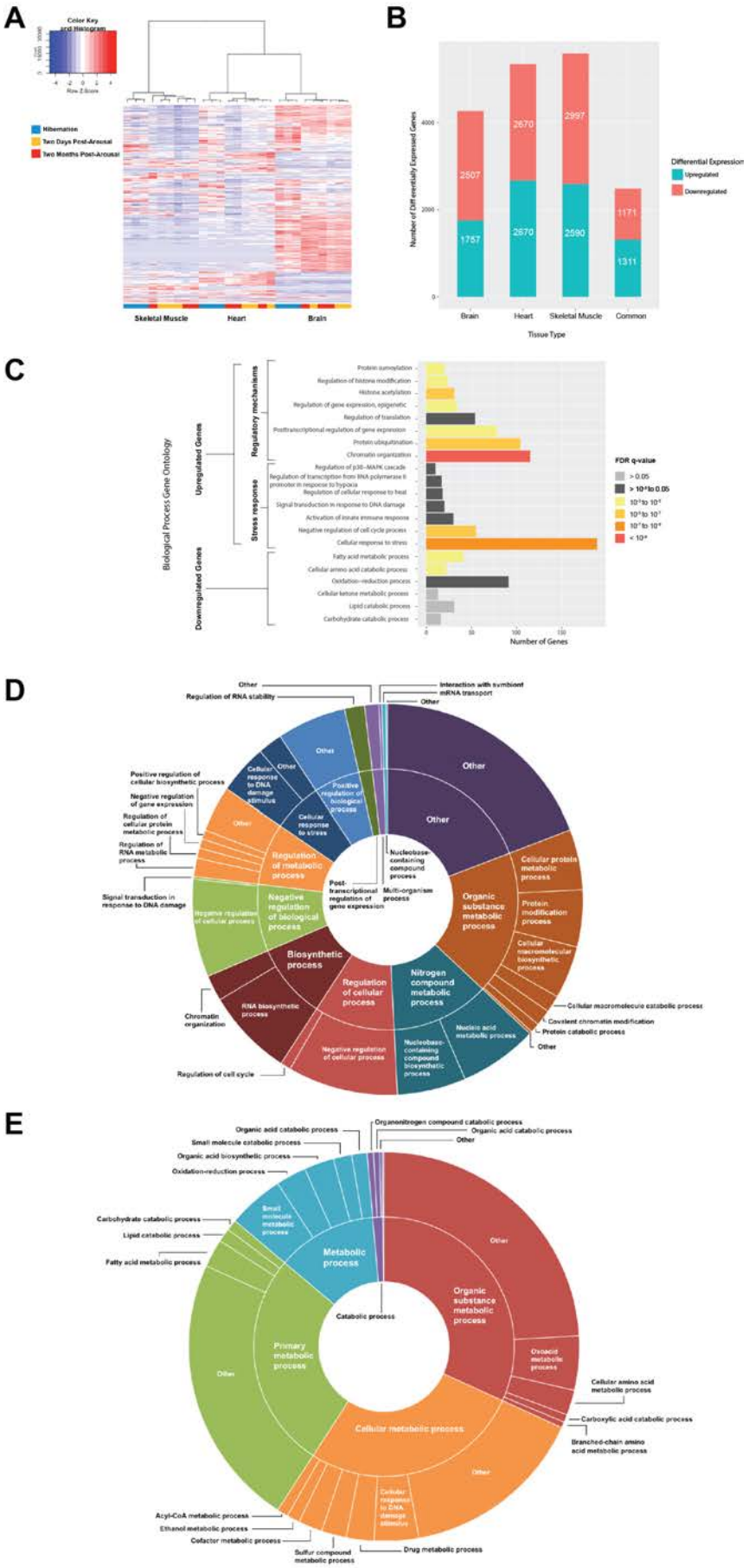

A
Color Key and Histogram
Row Z-Score
Hibernation
Two Days Post-Arousal
Two Months Post-Arousal
Skeletal Muscle
Heart
Brain
B
Number of Diff essentially Expressed Genes
2507
1757
2670
2670
2997
2590
1171
1311
Brain
Heart
Skeletal Muscle
Common
Tissue Type
Differential Expression
Upregulated
Downregulated
C
Biological Process Gene Ontology
Upregulated Genes
Downregulated Genes
Regulatory mechanisms
Stress response
Protein sumoylation
Regulation of histone modification
Histone acetylation
Regulation of gene expression, epigenetic
Regulation of translation
Posttranscriptional regulation of gene expression
Protein ubiquitination
Chromatin organization
Regulation of p38-MAPK cascade
Regulation of transcription from RNA polymerase II promoter in response to hypoxia
Regulation of cellular response to heat
Signal transduction in response to DNA damage
Activation of innate immune response
Negative regulation of cell cycle process
Cellular response to stress
Fatty acid metabolic process
Cellular amino acid catabolic process
Oxidation-reduction process
Cellular ketone metabolic process
Lipid catabolic process
Carbohydrate catabolic process
0
50
100
150
Number of Genes
FDR q-value
> 0.05
> 10⁻² to 0.05
10⁻³ to 10⁻²
10⁻⁴ to 10⁻³
10⁻⁵ to 10⁻⁴
< 10⁻⁵
D
Other
Regulation of RNA stability
Interaction with symbiont
mRNA transport
Other
Other
Other
Other
Cellular response to DNA damage stimulus
Other
Positive regulation of biosynthetic process
Negative regulation of gene expression
Regulation of cellular protein metabolic process
Regulation of RNA metabolic process
Other
Positive regulation of biological process
Cellular response to stress
Regulation of metabolic process
Signal transduction in response to DNA damage
Negative regulation of cellular process
Negative regulation of biological process
Post-transcriptional regulation of gene expression
Multi-organism process
Nucleobase-containing compound process
Organic substance metabolic process
Cellular protein metabolic process
Protein modification process
Cellular macromolecular biosynthetic process
Biosynthetic process
Regulation of cellular process
Nitrogen compound metabolic process
Cellular macromolecule catabolic process
Covalent chromatin modification
Protein catabolic process
Other
Chromatin organization
RNA biosynthetic process
Nucleic acid metabolic process
Negative regulation of cellular process
Nucleobase-containing compound biosynthetic process
Regulation of cell cycle
E
Organic acid catabolic process
Small molecule catabolic process
Organic acid biosynthetic process
Oxidation-reduction process
Organonitrogen compound catabolic process
Organic acid catabolic process
Other
Carbohydrate catabolic process
Lipid catabolic process
Fatty acid metabolic process
Small molecule metabolic process
Metabolic process
Other
Organic substance metabolic process
Catabolic process
Primary metabolic process
Other
Oxoacid metabolic process
Cellular amino acid metabolic process
Carboxylic acid catabolic process
Branched-chain amino acid metabolic process
Cellular metabolic process
Other
Cellular response to DNA damage stimulus
Acyl-CoA metabolic process
Ethanol metabolic process
Cofactor metabolic process
Sulfur compound metabolic process
Drug metabolic process

Figure 2-1. Differential gene expression and gene ontology enrichment analysis. (A) Heatmap of the 3,000 most highly expressed genes in all 27 samples with hierarchical clustering of samples. Each column represents a sample, and each row represents a gene. Each tile in the heatmap shows the normalized expression of a gene (Z-score), which was calculated by subtracting the mean expression value (counts per million) of a gene across all samples from the sample specific expression value, then divided by the standard deviation of the mean expression value of the gene. Hierarchical clustering and the dendrogram were calculated using Ward's method. Colour key shows Z-score, with blue indicating lower expression and red indicating higher expression compared to the mean across all samples. (B) Bar plot of the number of differentially expressed genes during hibernation as calculated in EdgeR (Log2 fold change > 0.585 – i.e. 1.5-fold change – and FDR < 0.05). The number of differentially expressed genes are overlaid on the bars. (C) Bar plot of selected enriched gene ontology (GO) terms of upregulated and downregulated genes common to all tissues during hibernation, with colour indicating FDR (q-value) of the GO term. (D) Donut plot of significantly enriched (FDR < 0.05) biological process GO terms for upregulated genes common to all tissues in hibernating individuals. (E) Donut plot of significantly enriched biological process GO terms for downregulated genes common to all tissues in hibernating individuals. The size of each segment is relative to the number of genes that fall within the specific gene ontology in our dataset.

Considering the small differences in gene expression between two days post-arousal and two months post-arousal samples (**S2-1 Table**), two days post-arousal individuals were

excluded from the proteomic analysis. Across all individuals (hibernators and awake) in brain, 743 proteins were identified, with the brain-specific proteins MBP, NEFM, and ATP1A2 most abundant (**S2-2 Table**). Twenty-seven of these proteins were upregulated, and 31 downregulated. In heart, the most abundant proteins were the muscle-specific proteins ACT, CKM, and MYH15. Of the 535 proteins identified, 29 were upregulated, and 28 downregulated during hibernation. In skeletal muscle, 337 proteins were identified, with muscle-specific proteins (CKM, TPM2, and TNNI2) the most abundant. Twenty of these proteins were upregulated, and 16 downregulated during hibernation. Overall, the correlation between mRNA and protein expression was very limited. In brain, there were 54 differentially expressed genes in the proteome that were detected in the transcriptome. However, in the transcriptome only 14 (26%) correlated with the proteome (2 upregulated and 12 downregulated during hibernation). This low correlation was mirrored in heart. Of 50 differentially expressed genes in the proteome that were detected in the transcriptome, 15 (30%) were correlated (1 upregulated and 14 downregulated during hibernation). Finally, skeletal muscle displayed the lowest correlation between proteome and transcriptome. There were 33 differentially expressed genes in the proteome, with just two (6%) correlating with the transcriptome. This low correlation may be due to the relatively low number of identified genes in the proteome (primarily high abundance proteins) compared to the transcriptome. For example, in brain 10,000 genes were identified with a count per million (CPM) > 10, while only 735 proteins were identified. This reflects a mere 7% of transcripts that have associated proteomic data.

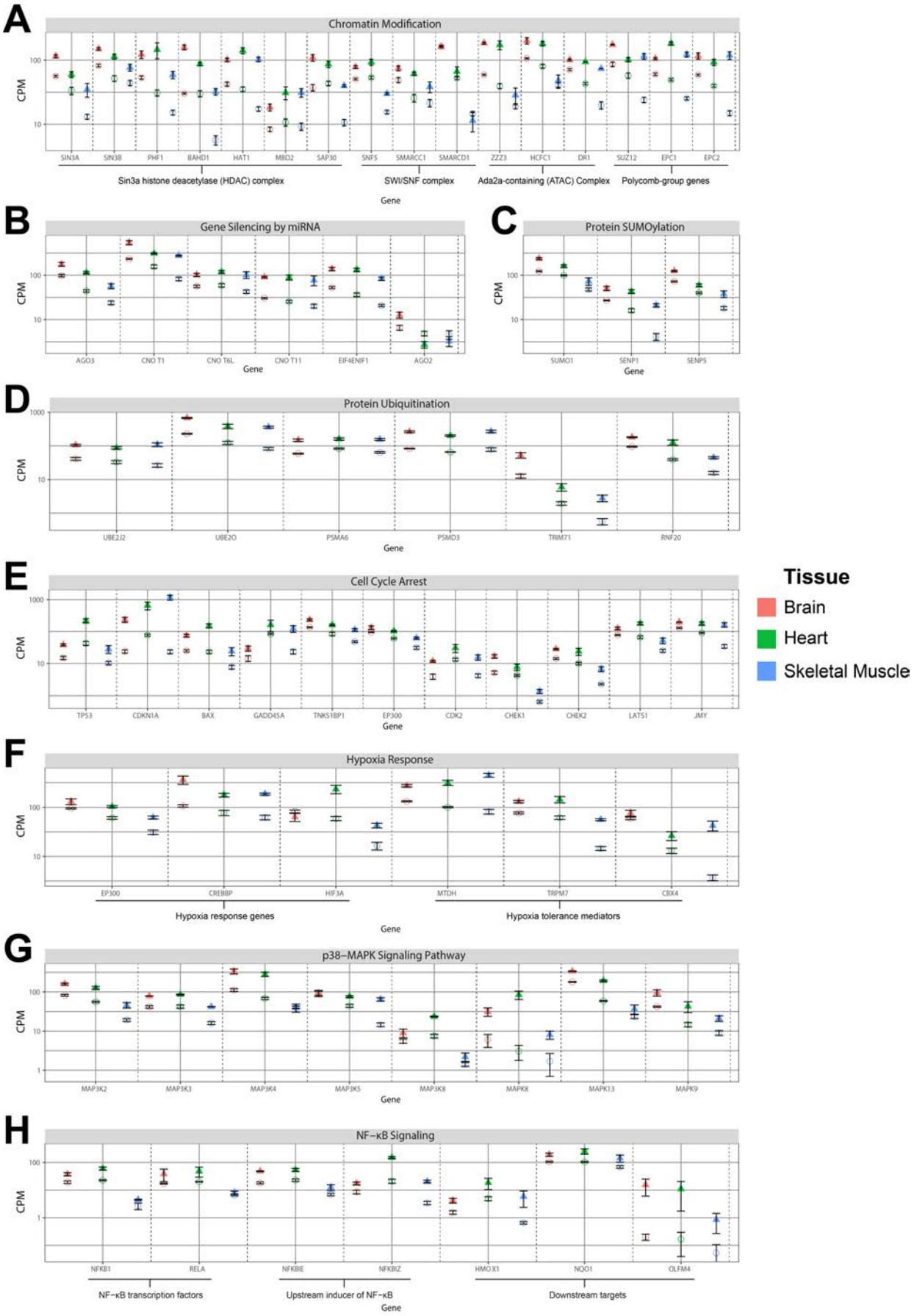
2.2.3.2 Biological processes common to all tissues examined

Gene ontology (GO) enrichment analysis of the 1311 common upregulated genes in hibernators identified 259 biological process GO terms that were significantly enriched

(FDR < 0.05; **Figure 2-1D and S2-3 Table**). These processes belonged to two major categories: 1) regulatory mechanisms of gene expression, protein translation and protein function; 2) cellular stress response and mitigation of stress severity (**Figure 2-1C**).

2.2.3.3 Regulation of gene expression

During hibernation, gene expression appears to be regulated at transcription, post-transcription, and post-translation across all tissues examined. Chromatin organization (GO:0006325) was enriched during hibernation (**Figure 2-1C and S2-3 Table**); with 115 upregulated genes that included members of epigenetic modifying complexes such as the Sin3a histone deacetylase (HDAC) complex, the SWI/SNF complex, the Ada2a-containing (ATAC) complex, and Polycomb-group genes (**Figure 2-2A**). Gene set enrichment analysis (GSEA) supported this finding, with enrichment of similar biological pathways, including chromatin organization (M13550) and regulation of gene expression, epigenetic (M16267), observed in during hibernation in all tissues (**S2-4 Table**). While chromatin modifying proteins were not differentially expressed in the proteomic data, changes in histone protein expression were evident across all tissues. H1F0 and HIST1H1D were upregulated during hibernation in brain (**Figure 2-3C**). In contrast, these two proteins were downregulated during hibernation in heart, whereas H4 was upregulated (**Figure 2-3G**). In skeletal muscle, H4 and H2B1C were both downregulated during hibernation (**Figure 2-3I**).


A
Chromatin Modification
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ZZZ3
HCFC1
DR1
SUZ12
EPC1
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Sin3a histone deacetylase (HDAC) complex
SWI/SNF complex
Ada2a-containing (ATAC) Complex
Polycomb-group genes
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Gene Silencing by miRNA
AGO3
CNOT1
CNOT6L
CNOT11
EIF4ENIF1
AGO2
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SENP1
SENP5
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Protein Ubiquitination
1000
UBE2J2
UBE2O
PSMA6
PSMD3
TRIM71
RNF20
E
Cell Cycle Arrest
TP53
CDKN1A
BAX
GADD45A
TNKS1BP1
EP300
CDK2
CHEK1
CHEK2
LATS1
JMY
F
Hypoxia Response
EP300
CREBBP
HIF3A
MTDH
TRPM7
CBX4
Hypoxia response genes
Hypoxia tolerance mediators
G
p38−MAPK Signaling Pathway
1
MAP3K2
MAP3K3
MAP3K4
MAP3K5
MAP3K8
MAPK8
MAPK13
MAPK9
H
NF−κB Signaling
NFKB1
RELA
NFKBIE
NFKBIZ
HMOX1
NQO1
OLFM4
NF−κB transcription factors
Upstream inducer of NF−κB
Downstream targets
Tissue
Brain
Heart
Skeletal Muscle

Figure 2-2. Differential expression of genes within enriched pathways during hibernation shared by all examined tissue. Mean (across biological replicates, ± 1 standard error) expression, measured in counts per million (CPM), of genes in brain (red), heart (green), and skeletal muscle (blue). Expression is shown for hibernators (triangles) and non-hibernators (circles). Panels (A) to (D) display genes related to gene expression regulatory mechanisms. Panels (E) to (H) display genes related to stress responses. The genes are from the following biological processes: (A) chromatin modification, (B) gene silencing by miRNA, (C) protein SUMOylation, (D) protein ubiquitination, (E) cell cycle arrest, (F) hypoxia response, (G) p38-MAPK signalling pathway, and (H) NF-κB signalling. All plotted on a $\log_{10}$ scale.

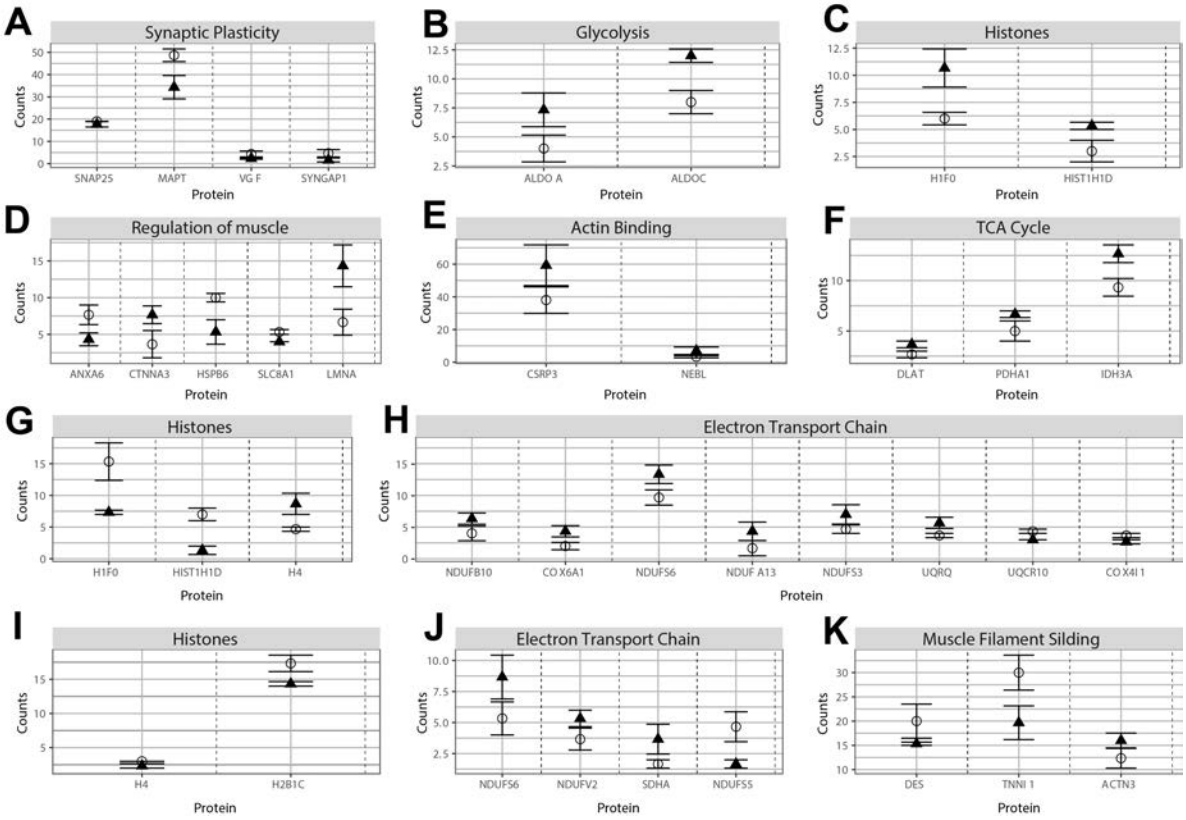

Figure 2-3. Differentially expressed proteins discovered between hibernators and two months post-arousal individuals within tissues. Mean (across biological replicates, ± 1 standard error) normalized spectral counts of proteins. Expression is shown for hibernators (triangles) and non-hibernators (circles). Panels (a) to (c) display differentially expressed proteins in brain; panels (d) to (h) display differentially expressed proteins in heart; and panels (i) to (k) display differentially expressed proteins in skeletal muscle. See **S2-3 Table** for fold-changes and full list of protein counts.

Regulation of translation (GO:0006417; 51 genes) was enriched during hibernation, and there were 18 genes upregulated during hibernation that modulate gene expression by miRNAs (GO:0060964; **S2-3 Table**). GSEA reinforced this enrichment (gene silencing by RNA; M16422) in all tissues (**S2-4 Table**). Importantly, these included genes required for miRNA-mediated translational repression (**Figure 2-2B**). Notably, the cleavage-

competent RNA-induced silencing complex (RISC) subunit *AGO2* (which results in mRNA degradation) was not differentially expressed (false discovery rate (FDR) > 0.05) between hibernators and non-hibernators. Finally, during hibernation there were enrichments for both protein SUMOylation (GO:0016925; 20 genes) and ubiquitination (GO:0016567; 104 genes; **Figure 2-2C and D; S2-3 Table**); an observation also supported by the GSEA in all tissues (**S2-4 Table**)

2.2.3.4 Response to stress

Genes associated with oxidative stress, hypoxia, DNA damage and heat shock pathways were upregulated during hibernation in all examined tissues (**Figure 2-1C**), along with 55 genes associated with negative regulation of cell cycle processes (GO:0010948; **S2-3 Table**). Eleven of these 55 genes are important in the regulation of p53-mediated cell cycle arrest (**Figure 2-2E**), including *TP53* and *CDKN1A*. Additionally, the p53-dependant G1 DNA damage response (M770) reactome pathway was highly enriched in all three tissues during hibernation (**S2-4 Table**). Genes that regulate transcription in response to hypoxia (GO:0061418) were also upregulated, and included the critical hypoxia response genes *EP300*, *CREBBP*, and *HIF3A*. A further three genes important for mediating hypoxia tolerance (*MTDH*, *TRPM7*, and *CBX4*) were also upregulated (**Figure 2-2F**).

The p38 mitogen activated protein kinase (MAPK) signalling cascade is responsive to various environmental stressors [208]. Ten genes within this signalling cascade (GO:1900744), including three MAP3Ks (MAP kinase kinase kinase), were upregulated in all tissues during hibernation. A further two MAP3Ks and three MAPKs were also upregulated in tissues of hibernators (**Figure 2-2G**).

Although undetected in the GO enrichment analyses, GSEA revealed that NF-κB signalling (M13738) was enriched during hibernation in all tissues (**S2-4 Table**). Specifically, seven genes within the NF-κB signalling pathway; a central regulator of oxidative stress response [209], were upregulated during hibernation. These included NF-κB transcription factors, upstream inducers of NF-κB signalling, and downstream target genes known to alleviate oxidative stress (**Figure 2-2H**).

2.2.3.5 Modulation of metabolism

Modulation of metabolic genes is a common feature in hibernators [31, 175, 210-215]. GO analysis of the 1171 common downregulated genes revealed 44 enriched biological processes (**Figure 2-1E and S2-1 Table**). This observation is directly supported by the GSEA, where the majority of enriched biological pathways post-arousal were related to metabolism (**S2-4 Table**). Enriched biological processes were predominantly related to metabolism, including lipid catabolic processes (31 genes – GO:0016042), oxidation-reduction processes (91 genes – GO:0055114), and carbohydrate catabolic processes (16 genes – GO:0016052; **S2-3 Table**). Furthermore, we observed a downregulation of three key ketone metabolic genes (*BDH2*, *ACAT1*, and *OXCT1*), which are necessary for metabolism when liver glycogen is depleted (**S2-1 Table**). Downregulated carbohydrate catabolism genes were predominantly related to glycoprotein and glycolipid metabolism (*NEU1*, *NEU2*, *ENOSF1*, and *NAGA*), glycosyl metabolism (*AGL* and *MAN2C1*), and galactose metabolism (*GALE* and *GALT*; **S2-1 Table**).

Enriched GO terms of common upregulated genes during hibernation related to metabolism were predominantly regarded carbohydrate metabolism, including regulation of carbohydrate metabolic process (29 genes – GO:0006109) and regulation of

gluconeogenesis (11 genes – GO:0006111). Specifically, this included *PFKFB3*, which stimulates glycolysis, *GSK3A*, which controls glycogen synthesis, and *FBP1*, the rate limiting enzyme of gluconeogenesis.

Corroborating our RNA-seq results, the proteomic analysis revealed differential expression of proteins involved in metabolic processes (particularly glucose metabolism) during hibernation in all tissues. In brain, two proteins upregulated during hibernation (ALDOA and ALDOC) are critical enzymes in glycolysis (**Figure 2-3B**). In heart, six upregulated proteins (NDUFB10, COX6A1, NDUFS6, NDUFA13, NDUFS3, and UQRQ), and two downregulated proteins (UQCR10 and COX4I1) are important in the respiratory electron transport chain. Additionally, three upregulated proteins (DLAT, PDHA1, and IDH3A) are involved in the tricarboxylic acid (TCA) cycle. In skeletal muscle three proteins (NDUFV2, NDUFS6, and SDHA), important in the respiratory electron transport chain, were also upregulated during hibernation, whereas one (NDUFA5) was downregulated.

2.2.3.6 Tissue-specific responses during hibernation

GSEA revealed an enrichment for the Kyoto Encyclopedia of Genes and Genomes (KEGG) disease pathway Alzheimer's disease (H00056) in the brain post-arousal (**S2-4 Table**). Three genes downregulated during hibernation (*PS1*, *PS2*, and *APOE*) are linked to early onset Alzheimer's disease (**S2-1 Table**). Furthermore, five genes responsible for the phosphorylation of the microtubule associated protein tau (MAPT – an important protein in the central nervous system) were upregulated during hibernation (**Figure 2-4A**). Additionally, in the bearded dragon, we observed that two N-methyl-D-aspartate receptor (NMDAR) genes were differentially expressed during hibernation: *GRIN1* was

downregulated during hibernation, and *GRIN2B* was upregulated during hibernation (**Figure 2-4B**). Proteomic analysis revealed four proteins involved in synaptic plasticity (SNAP25, MAPT, VGF, and SYNGAP1) were downregulated in brain during hibernation (**Figure 2-3A**).

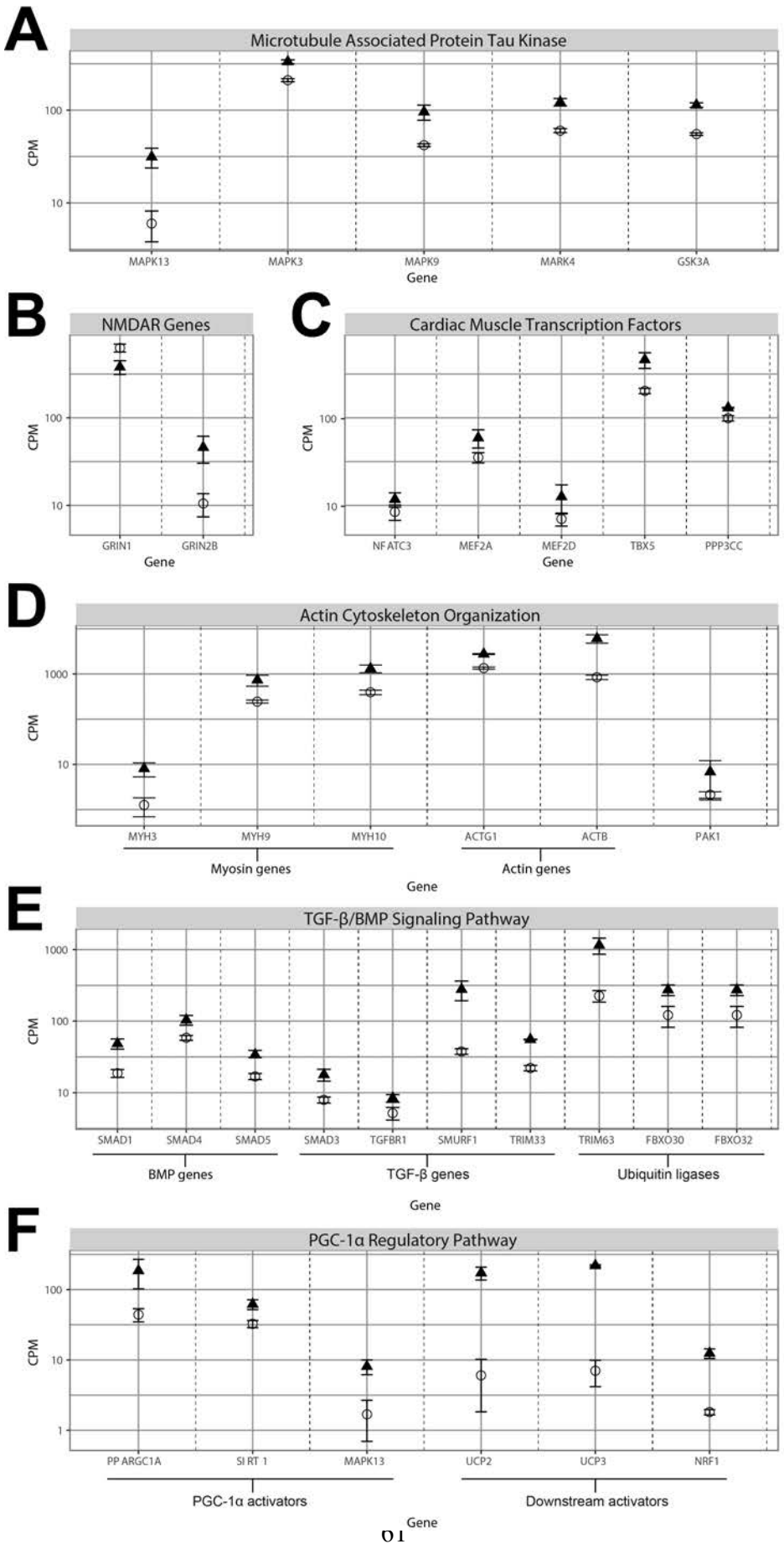
A
Microtubule Associated Protein Tau Kinase
CPM
100
10
MAPK13
MAPK3
MAPK9
MARK4
GSK3A
Gene
B
NMDAR Genes
CPM
100
10
GRIN1
GRIN2B
Gene
C
Cardiac Muscle Transcription Factors
CPM
100
10
NFATC3
MEF2A
MEF2D
TBX5
PPP3CC
Gene
D
Actin Cytoskeleton Organization
CPM
1000
10
MYH3
MYH9
MYH10
ACTG1
ACTB
PAK1
Myosin genes
Actin genes
Gene
E
TGF-β/BMP Signaling Pathway
CPM
1000
100
10
SMAD1
SMAD4
SMAD5
SMAD3
TGFBR1
SMURF1
TRIM33
TRIM63
FBXO30
FBXO32
BMP genes
TGF-β genes
Ubiquitin ligases
Gene
F
PGC-1α Regulatory Pathway
CPM
100
10
1
PPARGC1A
SIRT1
MAPK13
UCP2
UCP3
NRF1
PGC-1α activators
Downstream activators
Gene

Figure 2-4. Differential expression of genes within tissue-specific processes. Mean expression (across biological replicates, ± 1 standard error), measured in counts per million (CPM). Expression is shown for hibernators (triangles) and non-hibernators (circles). The genes are from the following brain-specific processes: (A) microtubule-associated protein Tau kinases, (B) N-methyl-D-aspartate receptor genes; heart-specific processes: (C) actin cytoskeleton organization, (D) cardiac muscle transcription factors; and skeletal muscle-specific processes: (E) the TGF-β signalling pathway/BMP pathway, and (F) the PGC-1α pathway. See **S2-1 Table** for fold-changes and false discovery rates.

During mammalian hibernation, transcription factors responsible for cardiac muscle development, and induction of cardiac hypertrophy, are important in maintaining cardiac function [88]. In bearded dragon, four cardiac transcription factors and *PPP3CC* (a cardiac hypertrophy regulator) were upregulated in heart during hibernation (**Figure 2-4C**). Furthermore, genes required for cardiac remodelling and associated with actin cytoskeleton modulation were upregulated during hibernation, including three myosin genes and two actin genes (**Figure 2-4D**). Proteomic analysis revealed 5 differentially expressed proteins (ANXA6, CTNNA3, HSPB6, SLC8A1, and LMNA) involved in regulating muscle system processes (**Figure 2-3D**), and 2 proteins (CSRP3 and NEBL) involved in actin binding (**Figure 2-3E**).

In skeletal muscle, the transforming growth factor beta-receptor (TGF-β) signalling pathway and bone morphogenetic protein (BMP) signalling pathway act antagonistically to balance muscle atrophy and hypertrophy [216]. We observed upregulation of three positive regulators of the BMP signalling pathway, and four positive regulators of the

TGF-β pathway. Three muscle atrophy-related ubiquitin ligases were also upregulated during hibernation (**Figure 2-4E**). The critical TGF-β gene myostatin (*MSTN*) was not differentially expressed between hibernating and awake animals. Finally, we observed upregulation of *PPARGCIA* (which encodes PGC-1α; the master regulator of mitochondrial biogenesis), along with two of its activators, and three downstream targets (**Figure 2-4F**); a process which is known to be important for prevention of muscle atrophy in mammals [217]. Three differentially expressed proteins identified by proteomic analysis are involved in muscle function (DES, TNNI1, and ACTN3); particularly muscle filament sliding (**Figure 2-3K**).

2.2.4 Discussion

Hibernation in reptiles is poorly studied compared to mammals. Beyond large-scale physiological responses, such as reduced heart and metabolic rate [218], the strategies common (and different) to hibernators from the two clades remain largely unknown. This study is the first to provide insight into the molecular pathways employed by a reptile during hibernation. We identified similarities between mammal and reptile hibernation, as well as responses that may be novel to the bearded dragon.

Recently, the use of steady state abundances of mRNA and proteins during hibernation has been scrutinized given that hibernation is a non-steady state condition. Some proteins do not function during hibernation as they do in steady state conditions, notably regulation of transcription by p53 [171]. However, for this study, we assume functional equivalence of biological processes during hibernation and after arousal. Additionally, transcriptomic and proteomic profiles were correlated to gain a multi-level understanding. Biological pathways (identified by gene ontology and gene set enrichment analyses) and

downstream targets, rather than specific genes, were focused on to gain a more nuanced representation of physiological responses during hibernation.

2.2.4.1 Control of gene expression

Evidence for multiple levels of gene regulation in hibernating bearded dragons was observed, which is unsurprising considering that alteration of gene expression is known to be critical for hibernation in mammals [5]. Accordingly, we identified upregulation of genes involved in RISC-mediated gene silencing in all tissues of hibernating bearded dragons (Figure 2-2B). Tissue-specific miRNA expression has been reported during hibernation in thirteen-lined ground squirrels [136, 138, 141], little brown bats (*Myotis lucifugus*) [105, 137, 139], monito del monte (*Dromiciops gliroides*) [106], and wood frog (*Rana sylvatica*) [219] (**Figure 2-5**).

Molecular mechanism	Bearded dragon	Ground squirrel	Fat-tailed dwarf lemur	Bear	Bat	Monito del monte	Frog
miRNA-mediated silencing	✓	✓ [138, 141]	✗	✗	✓ [105, 137, 139]	✓ [106]	✓ [219]
Upregulation of translational repression machinery	✓	✗	✗	✗	✗	✗	✗
Sin3A upregulation	✓	✓ [101]	✗	✗	✗	✗	✓ [102]
Cell cycle control	✓	✓ [10]	✗	✗	✗	✓[228]	✗
Oxidative stress response	✓	✓ [230]	✗	✗	✗	✓ [206, 231, 232]	✗
Modulation of metabolic genes	✓	✓ [210]	✓ [175]	✓ [211]	✓ [31, 212]	✓ [206, 213]	✓[214, 215]
Reduced NMDAR function in brain	✓	✓ [68]	✗	✗	✗	✗	✗
MAPT phosphorylation in brain	✓	✓ [73]	✗	✓ [73]	✗	✗	✗
Cardiac transcription factor upregulation in heart	✓	✓ [87]	✗	✗	✗	✗	✗
PAK1 upregulation in heart	✓	✗	✗	✗	✗	✗	✗
BMP + TGF-β modulation in skeletal muscle	✓	✓ [251]	✗	✗	✗	✗	✗
PGC-1α activation in skeletal muscle	✓	✓ [96]	✗	✗	✓ [255]	✗	✗

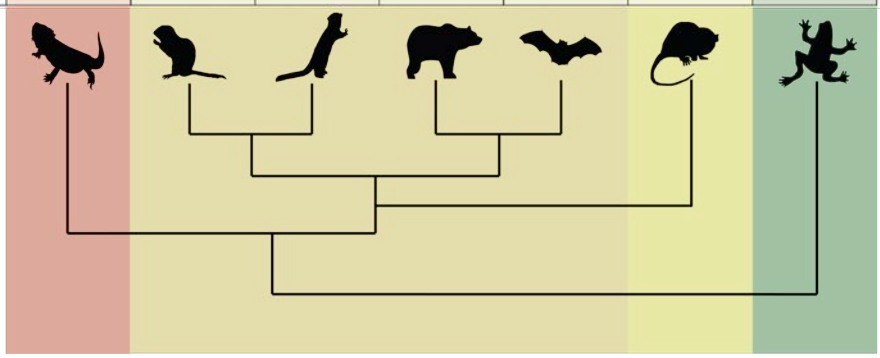

Figure 2-5. Summary of molecular mechanisms utilized during hibernation by the central bearded dragon and other hibernators. For each biological process, a tick indicates there is sufficient evidence to suggest that the process occurs, whereas a cross indicates a lack of, or insufficient, evidence that the process occurs rather than not occurring. Major vertebrate clades are identified by different colours (pink – reptile, yellow – mammal, and green – amphibian) on the phylogeny. All images used are the author's own work.

The CCR4-NOT complex is a master regulator of gene expression and is required for RISC-mediated translational repression via the recruitment of the translation initiation factor 4E-T [220-223]. During hibernation, we identified upregulation of three key

CCR4-NOT complex subunits, in addition to *EIF4ENIF1*, which encodes for 4E-T. Furthermore, in hibernating animals *AGO2* (necessary for cleavage-competent RISC) was not upregulated, instead *AGO3*; the catalytic subunit of non-cleavage-competent RISCs [224], was upregulated (**Figure 2-2B**). We propose that an enrichment of non-cleavage-competent RISC machinery during hibernation may represent an important gene regulatory pathway for bearded dragon hibernation.

Together with enhanced stability and polyadenylation of mRNAs [225], non-cleavage miRNA-mediated repression of mRNAs would allow for energy efficient regulation of gene expression. Upon arousal from hibernation reactivation of translation from mature mRNA molecules does not require immediate transcription and RNA processing. This would explain how bearded dragons are able to restore normal metabolic function promptly after arousal; such as if they are disturbed during hibernation, and after emergence from hibernation.

Restructuring of chromatin appears to be necessary for gene expression regulation during vertebrate hibernation. Increased expression of components of the Sin3A-HDAC complex is common to bearded dragons (**Figure 2-2A**), hibernating squirrels and frogs [101-104] (**Figure 2-5**). Increased expression of genes in the silencing complexes ATAC and SWI/SNF, along with Polycomb group proteins (**Figure 2-2A**), suggests that transcriptional control during hibernation requires the complicated interaction of many epigenetic modifiers. While differentially expressed chromatin remodelling proteins were not detected in the proteomic data, differential histone expression between hibernators and non-hibernators was apparent in all tissues (**Figure 2-3C, G and I**). The role of histone expression in phenotypic plasticity is largely unknown; however, histone

expression is important in genomic stability [226, 227], suggesting a potentially important role in hibernation.

An increased expression of small ubiquitin-like modifiers (SUMO) and ubiquitination genes was also observed during hibernation (Figure 2-2C and D), which are known to modulate protein function in mammalian hibernation (reviewed in [5]). Given the important role SUMOylation plays in cellular stress protection (reviewed in [150]), reversible post-translational regulation appears to be a universal mechanism involved in vertebrate hibernation and stress response.

2.2.4.2 Response to cellular stress

Cellular stress responses in the bearded dragon appear consistent with those of mammalian hibernators [10, 228] (**Figure 2-5**). Patterns of cell cycle arrest are a common observation during hibernation in thirteen-lined ground squirrels and in hypoxic red-eared slider turtles [10, 14] (**Figure 2-5**). In bearded dragon, the p53 stress response pathway may be important in mediating this response. During hibernation in ground squirrels, Pan et al. demonstrated that while p53 localizes to the nucleus, recruits RNA polymerase II and binds DNA, the lack of target gene activation suggests that p53 does not function equivalently during hibernation [171]. However, in bearded dragons, critical target genes (including *CDKN1A*, *BAX*, and *GADD45A*) were transcriptionally upregulated during hibernation in all tissues (**Figure 2-2E**), suggesting that p53 is indeed functional. In bearded dragons, the oxidative stress response may be guided by the NF-κB stress response pathway; much like in mammalian hibernators [229-232] (**Figure 2-5**). Increased expression of key target genes that protect against oxidative damage (*HMOX1*,

NQO1, and *OLFM4*) during hibernation suggests active protection from the sudden upsurge of reactive oxygen species that follows metabolic arousal from hibernation.

Our data implies that increased neuroprotection in brain during hibernation in bearded dragons may be governed by reduced N-methyl-D-aspartate receptor (NMDAR) function, ultimately preventing excitotoxicity: neuronal death by over-activation of glutamate receptors [67]. Downregulation of the NMDAR NR1 subunit gene *GRIN1* suggests lowered abundance of NMDARs at synapses, thus reducing capacity for excitotoxicity (**Figure 2-4B**).

Increased expression of MAPT kinases in the brain could result in hyperphosphorylation of MAPT (**Figure 2-4A**); a process that occurs in some hibernating mammals [73] (**Figure 2-5**) and is rapidly reversed upon arousal [72, 74, 233]. Phosphorylated MAPT has reduced affinity for microtubules, which is suggested to cause disruption of NMDAR anchoring and, therefore, neuroprotection (reviewed in [71]). This process is proposed to protect against excitotoxicity in hibernating ground squirrels [68, 234], anoxia-tolerant turtles [68, 234], and hypoxia and ischemia tolerance in the brains of rats and piglets [235-237]. Notably, MAPT-deficient mice are protected from excitotoxic brain damage [238]. In bearded dragons, *MAPT* mRNA expression did not vary significantly between hibernating and post-arousal time points, however, protein expression did (**Figure 2-3A**). The decreased abundance of MAPT protein in hibernating bearded dragons suggests that excitotoxicity prevention (via reduced receptor abundance and stability) is a critical protective measure in the brains of hibernators.

During mammal hibernation, cardiac hypertrophy increases contractile strength [80, 87]. Unlike hypertrophic cardiomyopathy disease in humans, cardiac hypertrophy in

hibernators is beneficial and quickly reversed upon arousal [87]. The cardiac-specific transcription factors with increased expression during bearded dragon hibernation (**Figure 2-4C**) have function in promoting cardiac hypertrophy [239], cardiac-specific gene expression [240], cardiac remodelling [241], and proper heart development [242]. Our data reflect those from hibernating ground squirrels (Figure 2-5) [37, 87, 89-91].

Cardiac hypertrophy requires modulation of the actin cytoskeleton and sarcomeres (the functional unit of muscle cells) [243]. This is mirrored by upregulation of actin and myosin genes during bearded dragon hibernation (**Figure 2-4D**). Maintenance of proper cardiac function was also revealed by the higher protein abundance of CSRP3 and NEBL during hibernation (**Figure 2-3E**). CSRP3 and NEBL bind actin and are important in maintaining muscle structure [244, 245], with mutations causing cardiomyopathy in mammals [246, 247].

Unique to bearded dragons, the important actin cytoskeletal gene *PAK1* was upregulated in heart during hibernation (**Figure 2-5D**). PAK1 regulates excitability and contractibility of cardiomyocytes (reviewed in [248]); with over-expression improving cardiac function in mice (reviewed in [249]), and deletion worsening hypertrophic cardiomyopathy [250]. Modulating actin organization and structure appears crucial for protecting cardiac function during hibernation.

In hibernating individuals, we observed upregulated pathways involved in prevention of skeletal muscle atrophy. The transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signalling pathways are antagonistic; they act to induce skeletal muscle atrophy and hypertrophy, respectively [216]. The increased expression of positive regulators of both pathways during hibernation is counterintuitive (**Figure 2-4E**).

However, both pathways modulate common targets (e.g. SMAD4 and the Akt/mTOR signalling cascade), and it has been suggested that normal maintenance of muscle mass results from precise regulation of both pathways [216]. In ground squirrels, members of both the TGF-β and BMP signalling pathways are also upregulated during hibernation (Figure 2-5) [251].

PGC-1α (encoded by *PPARGC1A*) is critical in muscle remodelling and mitochondrial biogenesis [252, 253]. Genes that activate PGC-1α have increased expression during hibernation in bearded dragons, as do downstream targets (**Figure 2-4F**). High levels of PGC-1α reduces muscle atrophy in non-hibernators (reviewed in [217]) by maintaining mitochondrial function, limiting inflammatory responses, and reducing ROS production and oxidative damage [254]. Induction of PGC-1α during hibernation may also mediate the switch from fast-twitch to slow-twitch muscle fibres [96, 255] (**Figure 2-5**), which is important for protecting the muscle from fatigue post-arousal. Upregulation of genes within the PGC-1α pathway during hibernation suggests that this process is occurring in bearded dragon (**Figure 2-4F**). Moreover, the increased abundance of proteins within the mitochondrial respiratory chain during hibernation (**Figure 2-3J**) indicates preservation of mitochondrial function. We propose that increased expression of genes within the PGC-1α regulatory pathway contributes to resistance of skeletal muscle atrophy in hibernating animals.

2.2.5 Conclusion

Here we conducted the first transcriptional profiling and proteomic analysis of a reptile during hibernation and post-arousal from hibernation. There was evidence of neuroprotective strategies in the brain, maintenance of heart function via hypertrophy, and protection against skeletal muscle atrophy via increased antioxidant capacity and mitochondrial maintenance during hibernation. Many protective strategies we observed in hibernating bearded dragons were consistent with hibernating mammals, suggesting that there are limited solutions available to tolerate such extreme stress at the cellular level. However, bearded dragons had responses not previously detected in mammals, including the enrichment of non-cleavage competent RISC machinery during hibernation.

2.2.6 Methods

2.2.6.1 Animals and Tissue Collection

Central bearded dragons (*Pogona vitticeps*) were captive bred and housed at the University of Canberra under a protocol approved by the University of Canberra Animal Ethics Committee (CEAE17-08) and ACT Government License to Keep (K9640). Husbandry practices fulfil the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) sections 3.2.13 - 3.2.23. Commercial sources of vegetables, mice and live insects (crickets and cockroaches) were provided as food, with water available *ad libitum*. Cages were cleaned thoroughly monthly, with superficial cleaning done daily (removal of faecal matter and unused food, maintenance of clean water containers). Logs and small branches were provided as basking perches and cardboard boxes provided as retreats. Enclosures were lit by a fluorescent lamp, a strong UVB light source, and a floodlamp (as a heat source) on a variable light:dark (L:D) cycle: August – mid-June (13hL:11hD; 22°C), late June (2 weeks- 6hL : 18hD; 18°C) and winter hibernation (0hL:24hD; 12°C). For two weeks prior to hibernation, heat and light were reduced and animals were not fed. All heat and UV lights were turned off for 8 weeks and the facility temperature maintained at 12°C, which stimulated any animals remaining active to hibernate. The conditions of artificial hibernation are chosen to mimic those that occur during natural hibernation, in that ambient temperatures are dropped, and light availability reduced. Body temperatures of hibernating animals was the same as ambient temperature (12°C) due to the lack of access to heat sources. After arousal from hibernation, animals were subject to full summer conditions (13hL:11hD; 22°C). Body temperatures of animals was at least 22°C (ambient) with the addition of access to a heat source.

Whole brain, whole heart and femoral skeletal muscle tissue were collected from three individuals at three time points: late hibernation, two days post-arousal and two months post-arousal. All samples were used in the transcriptomic analysis, while only late hibernation and two months post-arousal samples were used in the proteomic analysis. All lizards were male. Tissues were collected immediately after euthanizing (lethal injection of sodium pentobarbitone 65mg/kg by caudal venepuncture), snap frozen in liquid nitrogen and stored at -80°C until RNA and protein extraction. All post-arousal animals were sacrificed between zeitgeber time (ZT) 3 and ZT5, where ZT0 is lights on and ZT13 is lights off. Hibernating animals were sacrificed between circadian time (CT) 3 and CT5, where CT0 is the same time of day as ZT0, however, without lights turning on.

2.2.6.2 RNA Preparation and Sequencing

Total RNA was extracted from 50 mg of each tissue. Tissue extracts were homogenized in TRIzol reagent (Thermofisher, Waltham, Massachusetts, USA) using T10 Basic ULTRA-TURRAX® Homogenizer (IKA, Staufen im Breisgau, Germany), and RNA purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. An on-column DNase digestion was performed with RNase-free DNase (QIAGEN, Hilden, Germany). For each sample, 5-10 μg of high integrity RNA (RIN > 8) was poly-A selected. Libraries were constructed with the Illumina TruSeq Total RNA Stranded RNA kit, and 76 bp single-ended reads were generated on the Illumina NextSeq 500 platform at the Ramaciotti Centre for Genomics (UNSW, Australia). All sequence data have been submitted to the NCBI short read archive under the BioProject ID PRJNA476034.

Raw read quality was analysed with FastQC (v0.11.5) [256]. Trimmomatic (v0.36) [257] was used to trim the reads to remove low quality bases with the following options: HEADCROP:12 CROP:62 SLIDINGWINDOW:4:15. Reads were mapped to the annotated reference genome of the central bearded dragon [166] with HiSat2 (v2.0.5) [258] using default options. HTseq-count (v0.9.1) [259] was used to count reads that overlapped genomic features with the following options: -s reverse -m union. Samples were normalized using the trimmed mean of M-values (TMM) method and differentially expressed genes was calculated with EdgeR (v3.20.8) [260] in a pairwise manner using the exact test method. Resultant P-values were adjusted using the Benjamini-Hochberg procedure to calculate FDR. Genes with a fold-change greater than 1.5 ($\log_2$ fold-change of 0.585) and FDR less than 0.05 were considered differentially expressed. Gene ontology enrichment analysis related to differentially expressed genes were conducted with GOrilla using the human database (GO term database last updated December 9th 2017) [261]. Unranked lists of upregulated and downregulated genes in each condition and tissues were compared to a background list. The background list only included genes that were expressed (greater than 10 counts per million) within each tissue. For differentially expressed genes common to all tissues, only genes expressed in all three tissues were included in the background list. Gene set enrichment analysis (GSEA) [262] was performed for each tissue using defaults settings. Gene sets (Collection 2: Kegg, Biocarta and Reactome; Collection 5: GO Gene sets for Biological Process, Molecular Function and Cellular Component) were downloaded from MSigDB (reference = https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4707969/). As with the differential gene expression analysis, the two post-arousal time points were collated as a single time point. All graphs were plotted with R (3.4.2) [263], RStudio (1.1.383) [264], and ggplot2 (2.2.1) [265].

2.2.6.3 Protein Extraction and Mass Spectrometry

Total protein was extracted from 50 mg of tissue. Tissue extracts were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA), cOmplete® and EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) using T10 Basic ULTRA-TURRAX® Homogenizer (IKA, Staufen im Breisgau, Germany). Protein concentrations were determined using a Qubit 2.0 Fluorometer (Thermofisher, Waltham, Massachusetts, USA).

Protein extracts were analysed at the Bioanalytical Mass Spectrometry Facility at the Mark Wainwright Analytical Centre (UNSW, Australia) using label-free quantification mass spectrometry using standard protocol. Briefly, samples were digested with Trypsin (MS Grade, Thermofisher) and separated by nanoLC using an Ultimate nanoRSLC UPLC and autosampler system (Dionex, Amsterdam, Netherlands). Samples (2.5 µl) were concentrated and desalted with a micro C18 precolumn with H_2O:CH_3CN (98:2, 0.1 % TFA) at 15 µl/min and a fritless nano column (75 µm x 15cm) containing C18-AQ media (Dr Maisch, Ammerbuch-Entringen Germany). Peptides were eluted using a linear gradient of H_2O:CH_3CN (98:2, 0.1 % formic acid) to H_2O:CH_3CN (64:36, 0.1 % formic acid) at 200 nl/min over 60 min. 2000 volts was applied to low volume titanium union and the tip positioned ~ 0.5 cm from the heated capillary (T=275°C) of an Orbitrap Fusion Lumos (Thermo Electron, Bremen, Germany) mass spectrometer. Positive ions were generated by electrospray and the Fusion Lumos operated in data dependent acquisition mode (DDA).

A survey scan m/z 350-1750 was acquired in the orbitrap (resolution = 120,000 at m/z 200, with an accumulation target value of 400,000 ions) and lockmass enabled (m/z 445.12003). Data-dependent tandem MS analysis was performed using a top-speed approach (cycle time of 2 seconds). MS2 spectra were fragmented by HCD (NCE=30) activation mode and the ion-trap was selected as the mass analyser. The intensity threshold for fragmentation was set to 25,000. A dynamic exclusion of 20 seconds was applied with a mass tolerance of 10 parts per million.

Peak lists were generated using Mascot Daemon/Mascot Distiller (Matrix Science, London, England) and imported into the database search program Mascot (version 2.6.0, Matrix Science). Search parameters were: Precursor tolerance 4 ppm and product ion tolerances ± 0.5 Da; Met (O) carboxyamidomethyl-Cys specified as variable modification, enzyme specificity was trypsin, with 1 missed cleavage possible. Peaks were searched against the reference genome of the central bearded dragon [166] and a non-redundant protein database from NCBI (Jan 2015).

Raw peak data were imported into Scaffold (Matrix Science, London, England) and analysed accordingly with default settings. Normalized peak lists were imported into R (3.4.2) [263] for analysis. Proteins were excluded if there was an average of less than three spectral counts across the biological replicates in both conditions. Proteins were considered differentially expressed if the standard error of the mean spectral counts of each condition (*i.e.* hibernation vs. post-arousal) did not overlap.

2.2.7 Declarations

2.2.7.1 Ethics approval and consent to participate

Experimentation using animals was approved by the University of Canberra Animal Ethics Committee (CEAE17-08) and are in accordance with ACT Government License to Keep (K9640). Husbandry practices fulfil the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) sections 3.2.13 - 3.2.23.

2.2.7.2 Availability of data and materials

RNA-seq data are available in the NCBI short read archive under the BioProject ID PRJNA476034 (http://www.ncbi.nlm.nih.gov/bioproject/476034). Mass spectrometry data are available at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011171 and 10.6019/PXD01117 (https://www.ebi.ac.uk/pride/archive/projects/PXD011171). Computer code for processing and analysing sequence and mass spectrometry data is available on request.

2.2.7.3 Competing interests

The authors declare that they have no competing interests.

2.2.7.4 Consent for publication

Not applicable.

2.2.7.5 Funding

The project was funded by internally allocated funds from UNSW Sydney and in part by a grant from the Australian Research Council (DP170101147) awarded to AG and PW.

The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

2.2.7.6 Authors' contributions

PDW, SW, DO and AG conceived and designed the study. AC and HP contributed to the design of the study. AC performed the experiments. AC performed the computational analysis of sequencing and mass spectrometry data. HP conducted Gene Set Enrichment Analysis of sequencing data. AG provided tissue samples. AC and PDW wrote the paper. All authors read and approved the final manuscript.

2.2.7.7 Acknowledgments

We thank Wendy Ruscoe of the animal facility at the University of Canberra for animal husbandry and collecting tissue samples, and Ling Zhong at the Bioanalytical Mass Spectrometry Facility (BMSF) at the Mark Wainwright Analytical Centre (MWAC) for assistance with the mass spectrometry.

2.2.8 Appendices

Supplementary files are not included in this chapter due to excessive length and can be accessed via the following DOI: 10.1186/s12864-019-5750-x or file sharing link: https://www.dropbox.com/sh/kblxtdlry23vbts/AAD2pKLaV8gNCkyXNv83pw3ta?dl=0 . Supplementary file legends are provided below.

Additional File 1: S2-1 Table. Differential gene expression of RNA sequencing. Full list of differentially expressed genes with FDR < 0.05 for brain, heart, skeletal muscle, and common genes as outputted from EdgeR. Log_2 fold change is relative to hibernation (*i.e.* > 1 Log_2FC is higher expression during hibernation).

Additional File 2: S2-2 Table. Gene ontology enrichment analysis data of differentially expressed genes. Full list of enriched biological pathway gene ontologies in upregulated and downregulated gene datasets in brain, heart, skeletal muscle, and common genes as outputted from GOrilla.

Additional File 3: S2-3 Table. Protein expression data. Full list of all proteins identified with label-free mass spectrometry by Scaffold.

Additional File 4: S2-4 Table. Gene Set Enrichment Analysis (GSEA) results of RNA sequencing data. GSEA results of RNA sequencing data, containing Biocarta, KEGG, Reactome, and Biological Pathway results.

3 The role of microRNAs in regulating hibernation

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3.1 The role of microRNAs in regulating adaptive responses in hibernating central bearded dragons

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Your contribution to writing the paper:

I wrote all manuscript drafts and generated all figures.

Comments:

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3.2 Manuscript submitted for publishing

The role of microRNAs in regulating neuroprotection in the brain of hibernating central bearded dragons.

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Key words: Hibernation, *Pogona vitticeps*, central bearded dragon, RNA sequencing, miRNA, epigenetics, neuroprotection

3.2.1 Abstract

Background: Hibernation is a remarkable physiological state employed by many animals that are exposed to limited food and adverse winter conditions. Both tissue-specific and organism wide changes in metabolism and cellular function are employed to endure otherwise fatal physiological stress. Controlling hibernation initiation, maintenance, and arousal requires precise regulation of gene expression; including gene silencing by microRNAs (miRNAs). miRNAs are small non-coding RNA molecules (18 to 22 nucleotides) that post-transcriptionally regulate gene expression by either cleaving mRNA, or by reducing translation efficiency. Here we profiled miRNA expression in the Australian central bearded dragon (*Pogona vitticeps*) using small RNA sequencing of brain, heart, and skeletal muscle from individuals in late hibernation and four days post-arousal.

Results: A total of 1,295 miRNAs were identified in the central bearded dragon genome. Of these, 631 were conserved with known human and/or anole miRNAs, and 664 were novel to the central bearded dragon. During hibernation, there were seven differentially expressed miRNAs (DEmiRs) in brain, twelve in heart, and three in skeletal muscle. When all tissues were compared between hibernating and aroused individuals, 23 DEmiRs were identified. Target mRNAs for DEmiRs were predicted and correlated with expression data (mRNA-seq) from matched tissue. Functional analysis of DEmiR targets revealed an enrichment of differentially expressed mRNAs involved in metabolic processes. In brain, neuroprotective pathways were identified as potential targets of post-transcriptional regulation by miRNAs.

Conclusions: Our data provide support to the hypothesis that miRNAs contribute to regulating the hibernation phenotype. Specifically, miRNAs appear necessary for modulating the shift in cellular metabolism during hibernation and regulating neuroprotection in the brain. Furthermore, our results support the hypothesis that preventing excitotoxicity is a vital process in protecting the integrity of the brain during hibernation. This study is the first small RNA sequencing investigation in a hibernating reptile and provides further insight into this remarkable ephemeral phenotype.

3.2.2 Background

Animals that hibernate undergo remarkable seasonal change that involves profound modifications in their physiology, morphology and behaviour. Despite general differences amongst species, the adaptive strategies are common to most known hibernators. Typically, hibernation leads to a drastic reduction in basal metabolic activity, oxygen consumption, heart rate and core body temperature. The lowering of body temperature globally reduces rates of macromolecule synthesis and degradation, to redirect energy expenditure towards management of physiological stress (reviewed in [266]).

Hibernators reprioritise cellular fuel sources, switching from glucose-based sources to triglycerides and fatty acids that are stored prior to hibernation [3]. Stored fats are catabolised via lipolysis, beta oxidation, and ketogenesis. In some cases, such as in hibernating reptiles like the Australian central bearded dragon (*Pogona vitticeps*), large amounts of stored glycogen (in the tail) are used in conjunction with stored triglycerides [162]. Physiological stress that is common to all tissues includes nutrient deficiency, compromised immune system, and hypoxic-, oxidative- and cold- stress. Tissue-specific stressors include excitotoxicity in the brain [71] and muscle atrophy in skeletal muscle [267].

Studies aiming to understand the molecular architecture of hibernation phenotype have demonstrated control at multiple levels of gene regulation: including epigenetic changes (DNA methylation and histone modification), gene silencing by microRNAs (miRNAs), and protein modifications [5, 268]. Amongst these, the role of miRNAs in response to

stress, such as during hibernation, is becoming increasingly evident [105, 106, 135-141, 269].

MicroRNAs are small non-coding RNA molecules ranging from 18 to 22 nucleotides that post-transcriptionally regulate gene expression. miRNAs are initially transcribed as primary miRNAs (pri-miRNAs) with unique secondary hairpin structures that are processed in the nucleus by the RNase Drosha into precursor miRNAs (pre-miRNAs). Pre-miRNAs are further processed, by the RNase Dicer in the cytoplasm, into functional mature miRNA (reviewed in [129]).

Mature miRNA sequences are often conserved within Metazoa with a substitution rate of 3.5%, half of that of 18S ribosomal DNA (7.3%) [131, 132]. In humans, miRNAs are known to target and regulate over 60% of protein-coding genes [130]. The 5' seed region of miRNAs (nt 2-8) complement with 3'-untranslated regions (UTRs) of target mRNAs [133], recruiting the RNA-induced silencing complex (RISC), which subsequently cleaves and degrades the mRNA transcript [129]. Furthermore, RISC is able to repress translation of mRNAs, without degradation, by modulating the binding of ribosomes and their associated proteins [134]. miRNA-mediated translational repression is potentially a rapid and energy efficient means of regulating gene expression during hibernation that may be particularly important for 'kickstarting' normal function after arousal [268].

In hibernating mammals there are changes in miRNA expression have a clear role in regulating shifts in metabolism [105, 106], resistance to atrophy of skeletal muscle [135, 139] and increased neuroprotection in brain [137]. The low temperatures that animals typically hibernate are also predicted to facilitate twice as many microRNA-mRNA interactions than at 37°C [270]. However, changes in miRNA dynamics remains obscure

in hibernators outside of mammals. The central bearded dragon (*Pogona vitticeps*) is a well-established model to study reptilian hibernation miRNA dynamics as its genome is sequenced [166], and hibernation can be induced in captivity by reducing temperature to that of the during the coldest months of the Australian year; from May to September, with ambient temperatures ranging from 5°C to 18°C.

Here we explored the small RNA profiles of six Australian central bearded dragons in matched brain, heart, and skeletal muscle at two time points; 1) late hibernation, and 2) two months post-arousal from hibernation. We identified miRNAs conserved with *Anolis carolinensis*; the closest related reptile with annotated miRNAs, and *Homo sapiens*, and discovered novel miRNAs in central bearded dragons. Differential expressed miRNAs were identified, as were their predicted target mRNAs. miRNA expression was correlated with gene expression [268] from data matched tissues and time points. This is the first investigation of miRNAs in a hibernating reptile and revealed key novel and conserved miRNAs involved in regulating the switch in cellular fuel sources and neuroprotection in the brain. Furthermore, this study is the first to use small RNA sequencing to investigate miRNA dynamics during the hibernation season in any animal.

3.2.3 Results

3.2.3.1 Prediction of microRNAs in the central bearded dragon

Across all tissues (brain, heart and skeletal muscle), time points (late hibernation and two months post-arousal), and individuals (n=3 per tissue type at both time points), 1,295 miRNAs were predicted with a miRDeep2 score of greater than 4 (equal to signal to noise ratio of 8.1:1) in at least one tissue type (**Figure 3-1A, S3-1 Table**). Of these, we detected 547 that had seed regions conserved with just human miRNAs, and 252 that had seed regions conserved with only anole miRNAs (hereinafter referred to as "conserved miRNAs"; **Figure 3-1A**). We observed 168 that were conserved with both human and anole. The remaining 664 predicted miRNAs did not share conserved seed regions with either human or anole miRNAs. As such, these miRNAs were considered novel.

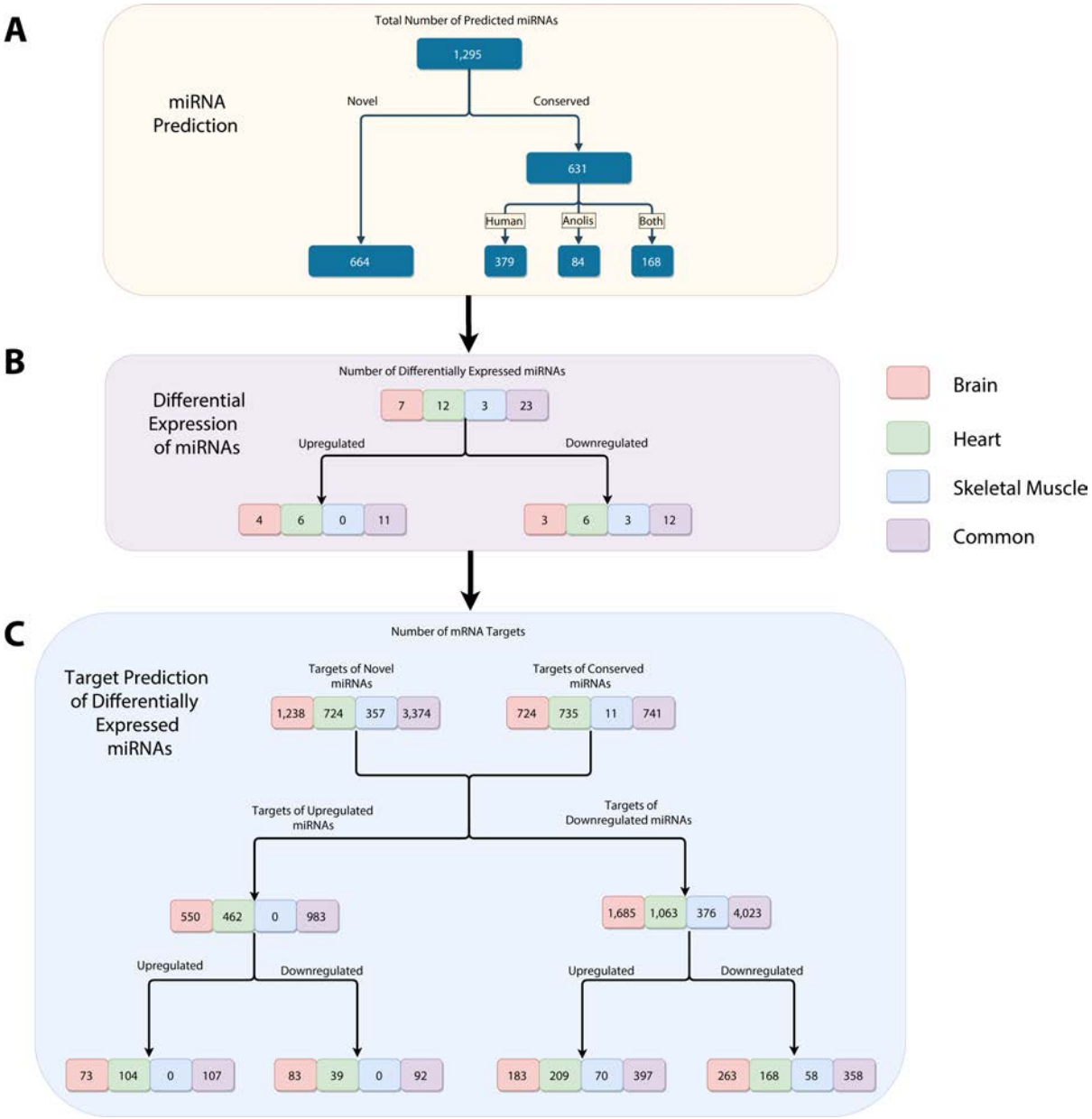

Figure 3-1. Summary of microRNA annotation, differential expression analysis and mRNA target prediction. Numbers of: **A** predicted miRNAs, **B** differential expressed miRNAs between hibernating and post-arousal individuals, and **C** predicted targets of differentially expressed miRNAs. Red is brain, green is heart, blue is skeletal muscle, and purple is when all tissues were analysed together.

3.2.3.2 Differential miRNA expression analysis

Differential miRNA expression analysis was performed on all conserved and novel miRNAs. Hierarchical clustering according to expression values of the 1,295 predicted miRNAs resulted in distinct groupings of tissue types (**Figure 3-2A**). Brain had the largest number of highly expressed miRNAs, followed by heart, and skeletal muscle. In no tissue was there clear clustering of hibernating or post-arousal individuals (**Figure 3-2A**).

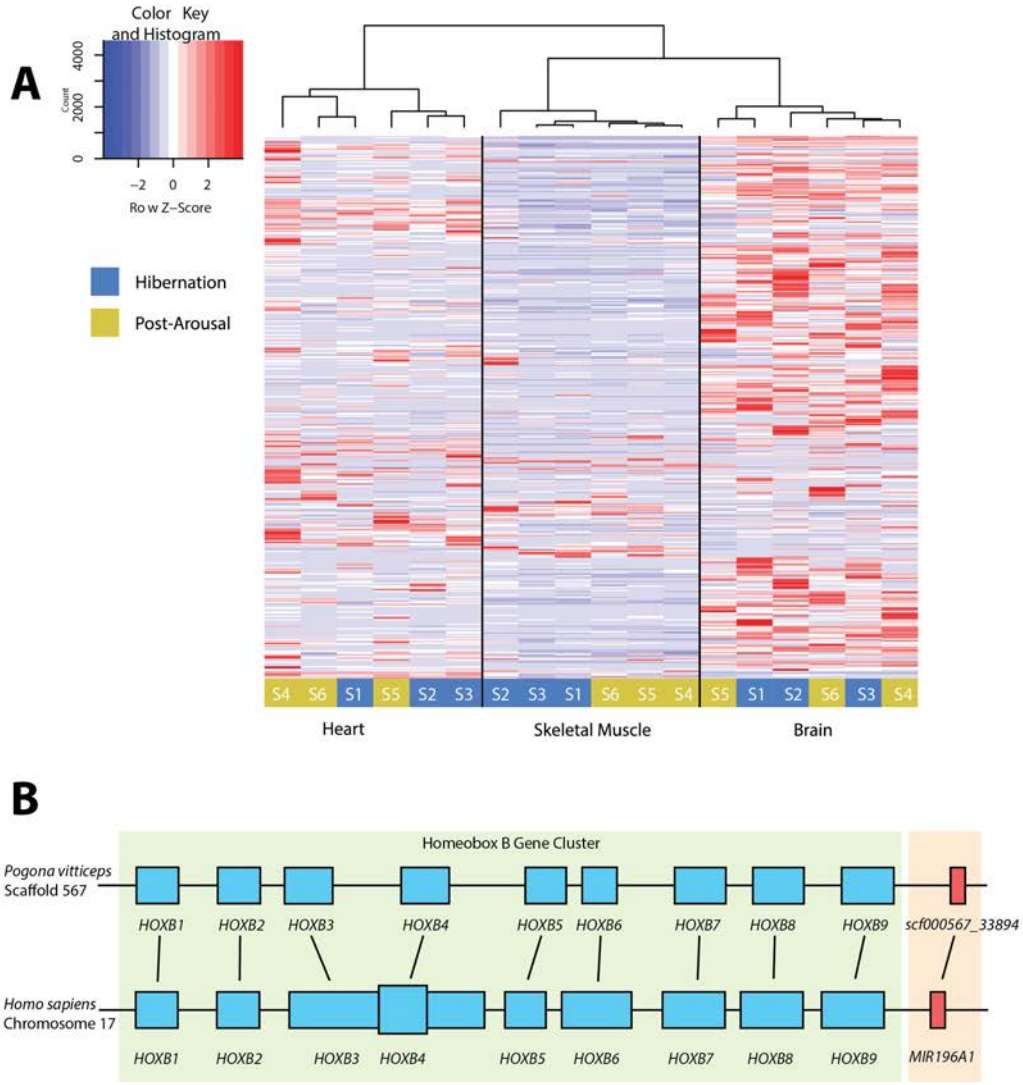

Figure 3-2. Differential miRNA expression and miRNA gene synteny. a Heatmap of expressed miRNAs in all 18 samples with hierarchical clustering. Each column represents a sample and each row a miRNA. The normalized expression of a miRNA (Z-score) within each sample was calculated by subtracting the mean expression across all samples from the sample specific expression value, then dividing by the standard deviation of the mean expression value. Hierarchical clustering and the dendrogram were calculated using Ward's method. Red Z-scores indicate higher expression and blue lower expression compared to mean expression across all samples. **b** Synteny analysis of mir-196 between *Pogona vitticeps* (scf000567_33894) and *Homo sapiens* (MIR196A1).

In brain, the highest expressed miRNA during both hibernation and post-arousal was miR-9-5p; an abundant and conserved miRNA in the brain of vertebrates [271, 272]. In both heart and skeletal muscle, miR-1-3p (a conserved and critical cardiac and skeletal muscle miRNA [273]) was the most abundant miRNA during hibernation and post-arousal.

The greatest number of differentially expressed miRNAs (DEmiRs) between hibernating and aroused animals was observed in heart, followed by brain, and then skeletal muscle (**Figure 3-1B, Table 3-1**). In heart and brain, 41.6% (5 out of 12) and 42% (3 out of 7) of DEmiRs were conserved with either human or anole miRNAs, whereas conserved DEmiRs were at 66.6% (2 out of 3) for skeletal muscle.

Table 3-1. List of all differentially expressed microRNAs with FDR < 0.05.

miRNA ID	miRNA	Log_2 Fold Change	Adjusted p-value	Synteny	Number of Predicted Targets	Differential Expression of Targets (per condition)
Brain						
scf000152_19859	Novel	6.527	0.02005973	N/A	402	↑ 73 ↓ 83
scf000119_5395	Novel	5.589	0.01114128	N/A	24	
scf000549_15459	miR-4696	3.513	0.01641398	No	108	
scf000258_20371	miR-1468-5p	1.393	0.00210684	No	16	
scf000478_4389	Novel	-1.122	0.00193561	N/A	176	↑183 ↓263
scf000260_12856	miR-149-3p	-2.547	0.01901295	No	610	
scf000063_9938	Novel	-4.244	0.01805405	N/A	899	
Heart						
scf000119_5395	Novel	5.784	0.00789498	N/A	24	↑ 104 ↓ 39
scf000314_20127	mir-203-3p	4.055	0.02801801	Yes	282	
scf000714_4624	Novel	3.683	0.02694264	N/A	263	
scf000019_10057	miR-338-3p	2.258	0.00789498	Yes	119	
scf000415_13898	miR-551-3p	1.959	0.02205359	Yes	8	
scf000258_20371	miR-1468-5p	1.544	0.00361614	No	16	

miRNA ID	miRNA	Log_2 Fold Change	Adjusted p-value	Synteny	Number of Predicted Targets	Differential Expression of Targets (per condition)
scf000052_33382	Novel	-1.439	0.04137763	N/A	252	↑ 209 ↓ 168
scf000478_4389	Novel	-1.584	3.35E-06	N/A	176	
scf001197_18393	Novel	-1.620	3.35E-06	N/A	126	
scf000567_33894	mir-196-5p	-2.621	0.02694264	Yes	308	
scf000172_9618	Novel	-5.671	0.02350143	N/A	53	
scf000433_24590	Novel	-5.924	0.02694264	N/A	148	
Skeletal Muscle						
scf000194_40008	mir-460b-5p	-2.673	0.02371199	Yes	11	↑ 58 ↓ 70
scf001197_18393	Novel	-1.325	0.02444514	N/A	239	
scf000568_104	mir-1306-3p	-0.996	0.00029401	Yes	126	
Common						
scf000777_10824	miR-5436-3p	6.064	0.0135961	No	138	↑ 107 ↓ 92
scf000094_1910	Novel	5.358	0.006831	N/A	135	
scf000119_5395	Novel	5.147	3.68E-08	N/A	24	
scf000152_19859	Novel	4.950	2.48E-05	N/A	402	
scf000121_1222	Novel	3.913	0.00915318	N/A	1	
scf000441_19211	Novel	3.898	0.00149224	N/A	39	
scf000513_11489	Novel	3.467	0.04490014	N/A	42	
scf000714_4624	Novel	3.467	1.61E-07	N/A	13	

miRNA ID	miRNA	Log_2 Fold Change	Adjusted p-value	Synteny	Number of Predicted Targets	Differential Expression of Targets (per condition)
scf000072_5856	Novel	3.131	0.0135961	N/A	68	
scf000549_15459	miR-4696	2.126	0.01145064	No	108	
scf000258_20371	miR-1468-5p	1.448	0.00017233	No	16	
scf000739_36870	Novel	-1.213	0.01292055	N/A	45	↑ 397 ↓ 358
scf000478_4389	Novel	-1.227	0.00149224	N/A	176	
scf000030_21650	Novel	-1.285	0.01979265	N/A	402	
scf000260_12856	miR-149-3p	-1.395	0.04167983	No	854	
scf001086_36659	miR-6809-3p	-2.566	0.0077862	No	309	
scf000063_9938	Novel	-3.311	0.00017233	N/A	676	
scf002320_34830	Novel	-3.321	0.00649737	N/A	155	
scf000567_33894	mir-196a-5p	-3.799	0.02233976	Yes	308	
scf000319_14315	Novel	-4.520	0.04450324	N/A	141	
scf000068_14290	miR-7481-3p	-5.228	0.00149224	No	159	
scf000134_8387	Novel	-6.434	0.01292055	N/A	398	
scf000164_27058	Novel	-6.492	0.0009095	N/A	401	

When the different tissues were treated as biological replicates at the same time point (hereinafter referred to as common), 23 miRNAs were differentially expressed between hibernating and post-arousal animals (**Figure 3-1B, Table 3-1**). Six DEmiRs were conserved with human and/or anole. The remaining were novel to bearded dragon.

3.2.3.3 Synteny conservation of known differentially expressed miRNAs

For conserved miRNAs, local gene synteny was assessed (**Figure 3-2B, Table 3-1**). Gene order adjacent to the bearded dragon miRNAs were compared with that of anole, chicken, and human. Both conserved skeletal muscle DEmiRs shared synteny between species, four of five conserved heart DEmiRs shared synteny, whereas no conserved brain DEmiRs shared synteny with any species (**Table 3-1**). Only one of six conserved DEmiRs common to all tissues shared synteny with any of the tested species.

3.2.3.4 Prediction of miRNA targets

Because 65.2% (30 of 45) of DEmiRs were novel, identification and prediction of target mRNAs predicted for sequence binding could uncover potential their biological relevance (summarised in **Figure 3-1C, S3-1 Figure**). Two tools were used for target prediction of novel miRNAs; miRanda [274] and RNA22 [275], with target mRNAs predicted by either tool being retained. For the conserved miRNAs, target mRNAs were obtained using multiMiR [276]; a collection of validated miRNA-targets. We predicted 1,238 unique targets of the four novel DEmiRs in brain (**Figure 3-1C, S3-1 Figure, S3-2 Table**), 724 unique targets of the 7 novel DEmiRs in heart, and 357 unique targets from the single novel DEmiR in skeletal muscle. For the 17 novel DEmiRs common to all tissues, 3,374 unique targets were identified.

3.2.3.5 Differentially Expression Analysis of Target Genes

RNA-seq and proteomic data from matched hibernating and post-arousal individuals [268] was used to assess the expression of DEmiR targets (**S3-3 Table**). Most target genes were detected in the mRNA expression data, however, only a small proportion of target genes were identified in the proteomic data. Canonically, miRNAs are well known to degrade target mRNAs, therefore, the downregulated targets of upregulated miRNAs during hibernation were of key interest, in addition to the upregulated targets of downregulated miRNAs.

In the brain transcriptome, the upregulated miRNAs during hibernation had 83 out of 550 (15.1%) targets downregulated, and 73 (13.3%) upregulated (**Figure 3-1C**). The downregulated miRNAs in brain had 183 out of 1,685 (10.9%) targets upregulated, and 263 (15.6%) downregulated. In the proteome, 37 target genes of upregulated miRNAs were identified, with 4 differentially expressed (2 upregulated and 2 downregulated; **S3-3 Table**), including MAP6 which was downregulated in both the transcriptome and proteome. Proteins of 69 target genes of the downregulated miRNAs were identified in brain during hibernation. Seven of these proteins were differentially expressed (4 upregulated and 3 downregulated). However, only *CNBP* was upregulated in both the transcriptome and proteome.

In heart, the upregulated miRNAs during hibernation had 39 out of 462 (8.4%) targets were downregulated, and 104 (22.5%) were upregulated (**Figure 3-1C**). The downregulated miRNAs in heart had 209 out of 1063 (19.7%) targets upregulated, and 168 out of 1063 (15.8%) downregulated. In the proteome, 15 target genes of upregulated miRNAs were identified; none of which were differentially expressed (**S3-3 Table**).

Proteins of 14 target genes of downregulated miRNAs were identified; 4 of which were differentially expressed (all downregulated).

In skeletal muscle, the downregulated miRNAs during hibernation had 58 out of 376 (20.4%) target genes upregulated and 70 (18.6%) downregulated (**Figure 3-1C**). In the proteome, only 5 targets of downregulated miRNAs were successfully identified; 2 of which were differentially expressed (1 upregulated, 1 downregulated; **S3-3 Table**).

The upregulated miRNAs common to all tissues had 92 out of 983 (9.4%) target genes downregulated, and 107 (10.1%) upregulated (**Figure 3-1C**). The common downregulated miRNAs had 397 out of 4,023 (9.9%) were upregulated, and 358 (8.9%) downregulated.

3.2.3.6 Functional Analysis of Differentially Expressed Targets

To assess potential functional capacity of differentially expressed miRNAs during bearded dragon hibernation, GO enrichment analysis of biological processes was performed on the differentially expressed targets of DEmiRs. Significantly enriched GO terms (p-value < 0.05) were observed in tissues (**S3-4 Table**).

3.2.3.6.1 Common to All Tissues

In hibernating individuals, upregulated targets of downregulated miRNAs were enriched for 82 diverse biological processes, including: regulation of RNA polymerase II (GO:0006357), chromatin organization (GO:0006325), histone modification (GO:0016570), protein modification by small proteins (GO:0070647), response to light stimulus (GO:0009416), and regulation of circadian rhythm (GO:0042752). Five miRNAs that target key glucose and fat metabolism genes were downregulated during

hibernation across all three tissues: scf000030_21650, miR-149-3p (scf000260_12856), miR-196a-5p (scf000567_33894), miR-6809-3p (scf001086_36659) and scf002320_34830. The targets of these genes include *PPARGC1A*, *CRTC1*, *CRTC2*, *FOXO1* and *PRKAG1*. These genes were upregulated during hibernation in all tissues with the exception of *PPARGC1A*, which was downregulated in brain [268].

miRNAs that target genes important in miRNA-mediated translational repression (*AGO3*, *CNOT1* and *CNOT11*) were downregulated during hibernation. *CNOT1* was targeted by scf000478_4389 and *CNOT11* was targeted by scf000567_33894. Interestingly, three miRNAs that targeted *AGO3* were downregulated: miR-7481-3p (scf000068_14290), scf000164_27058 and scf000478_4389.

3.2.3.6.2 Brain

During hibernation, downregulated target mRNAs of upregulated miRNAs were enriched for four GO terms, including membrane organization and double-stranded break repair (**S3-4 Table**). Upregulated targets of downregulated miRNAs were enriched for 38 GO terms. GO terms were related to regulation of gene expression, including protein modification by small protein, gene silencing by RNA, negative regulation of gene expression, and histone acetylation (**S3-4 Table**).

The novel bearded dragon miRNA scf000152_19859 was a particularly interesting DEmiR. It was expressed in hibernators, but undetectable in post-arousal brain. GO term analysis of all target genes of scf000152_19859 revealed enrichment for several neuronal and synaptic signalling processes; including regulation of AMPA receptor activity, regulation of short-term neuronal synaptic plasticity, and regulation of positive synaptic transmission (**S3-4 Table**). Two targets of this miRNA are *MAP6* and *PLEC*, which were

both downregulated at the protein level. *MAP6* mRNA was also downregulated. These two genes are involved in stabilization of microtubules [277, 278]. Furthermore, scf000152_19859 targets *CAMK2A* which was downregulated during hibernation at the mRNA level. *PARP1* mRNA*;* targeted by miR-4696 (scf000549_15459) was also downregulated.

3.2.3.6.3 Heart and Skeletal Muscle

During hibernation in heart, the downregulated targets of upregulated miRNAs were enriched for five GO terms, including glucose homeostasis (GO:0042593) and vitamin biosynthetic process (GO:0009110). In skeletal muscle of hibernating individuals, upregulated targets of the downregulated miRNA were enriched for regulation of metabolic processes (GO:0080090) and response to cold (GO:0070417).

In heart, upregulated targets of downregulated miRNAs were enriched for 17 biological processes, including regulation of gene expression (GO:0010468), and ERK1/ERK2 cascade (GO: 0070371). Within the ERK1/ERK2 pathway, three key genes were upregulated: *MAPK1*, *MYC*, and *MAP2K2*. *MAPK1* and *MYC* were targeted by mir-196a-5p (*scf000567*_33894), and *MAP2K2* by scf000030_21650.

3.2.3.7 Differentially Expressed miRNA-Target Network Analysis

Network analysis of differentially expressed miRNAs and their target mRNAs was performed with Cytoscape (v3.7.1) [279] (**Figure 3-3**). Differentially expressed miRNAs generally shared many common target genes with one another. However, when only differentially expressed target mRNAs were considered, miRNAs shared few target mRNAs. Apart from commonly upregulated miRNAs (**Figure 3-3F**), upregulated

miRNAs did not share downregulated targets in any tissue. The downregulated miRNAs in brain and heart shared many upregulated targets during hibernation (**Figure 3-3B, D**). This was also observed for commonly downregulated miRNAs during hibernation (**Figure 3-3G**).

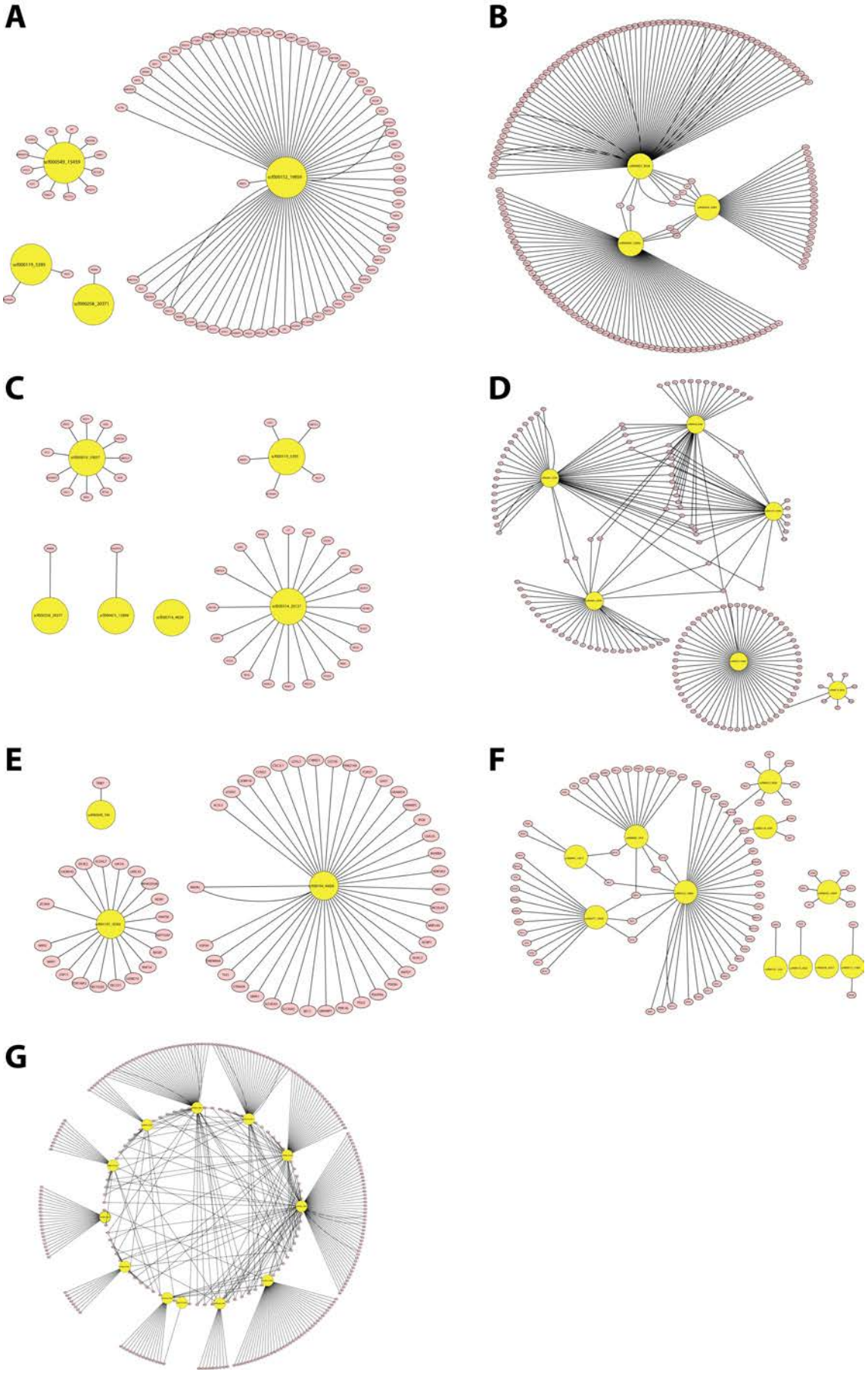
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Figure 3-3. **miRNA target mRNA networks**. Brain: **a** Upregulated miRNAs and their downregulated targets, **b** downregulated miRNAs and their upregulated targets. Heart: **c** Upregulated miRNAs and their downregulated targets, **d** downregulated miRNAs and their upregulated targets. Skeletal muscle: **e** Downregulated miRNAs and their upregulated targets. Common to all tissues: **f** upregulated miRNAs and their downregulated targets, **g** downregulated miRNAs and their upregulated targets. Yellow nodes represent differentially expressed miRNAs and pink nodes represent differentially expressed target mRNAs.

3.2.4 Discussion

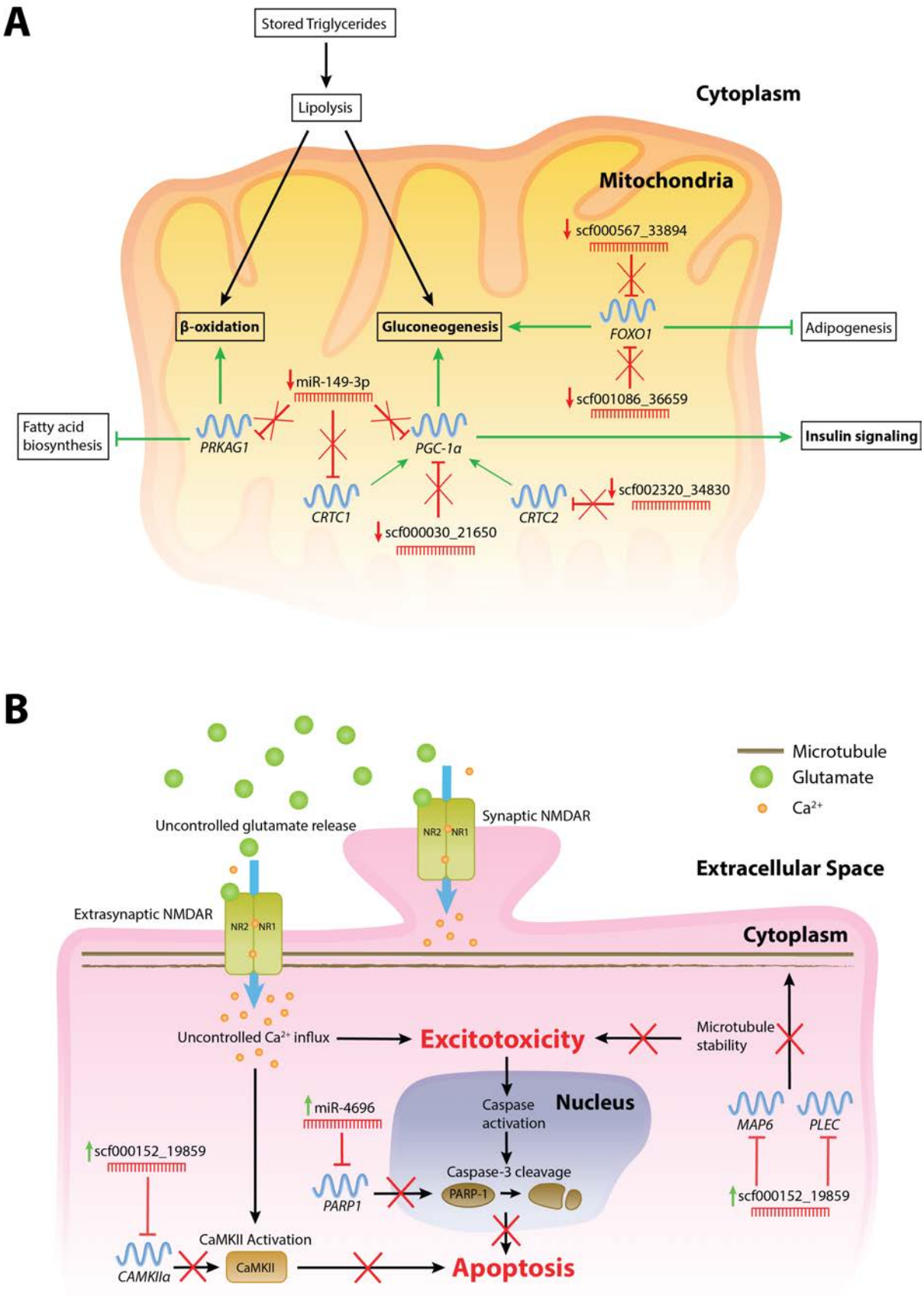
The drastic changes in cellular physiology during hibernation necessitates the need for precise control of gene expression. In mammals, there is increasing evidence for the importance of miRNAs in maintaining correct gene product abundance during hibernation [105, 106, 136-141]. However, the role of miRNAs in reptilian hibernators has yet to be examined. For the first time, we have identified conserved and novel miRNAs in the central bearded dragon genome. We show that changes in miRNA expression correlates with change of target mRNA abundance in heart and skeletal muscle, and both mRNA and protein abundance in brain during hibernation. Our results support the idea that multi-level regulation of gene expression is required for modulating hibernation and elucidates specific processes that miRNAs modulate during bearded dragon hibernation.

In bearded dragon, differential expression of miRNAs appears to be largely tissue-specific. However, 23 miRNAs were identified as differentially expressed when all tissues were compared between the two time points. Considering the drastic changes in mRNA expression [268], a surprisingly small number of miRNAs displayed differential expression. However, miRNAs can target multiple mRNAs [280], so hibernation may only require modulation of a few critical miRNAs. Half (664 out of 1,295) of the differentially expressed miRNAs were not annotated in any other species.

3.2.4.1 miRNA expression during hibernation correlates with shifts in cellular metabolism

A gene ontology enrichment for insulin resistance was observed for upregulated targets of downregulated miRNAs during hibernation (**S3-4 Table**). Insulin resistance is a

hallmark of mammalian hibernation (reviewed in [202]). It was proposed that insulin resistance occurs prior to hibernation as a mechanism to store excess body fat, which is subsequently reversed during hibernation. During hibernation the upregulated targets included several key glucose and fat metabolism regulators such as: *PPARGC1A*, *CRTC1*, *CRTC2*, *FOXO1* and *PRKAG1* (**Figure 3-4A**). *PPARGC1A*, which encodes PGC-1α, is a gene critical for regulating energy metabolism and mitochondrial biogenesis. Activation rescues insulin signalling in insulin-resistant mice and induces gluconeogenesis (the production of glucose from non-carbohydrate sources – reviewed in [281]). CRTC1, CRTC2 and PRKAG1; the regulatory subunit of AMPK, are all coactivators of PCG-1α. During energy depletion or stress, AMPK is activated and, together with PGC-1α, activates fatty acid oxidation and increased mitochondrial activity (reviewed in [282]) [283, 284]. FOXO1 interacts with PGC-1α to induce expression of gluconeogenic enzymes [285, 286], as well as acting as a negative regulator of adipogenesis [287]. miR-149-3p (scf000260_12856) targets *PPARGC1A*, *FOXO1* and *PRKAG1*. During hibernation the reduced expression of miRNAs that target these key metabolic genes may release gene repression to promote increased fatty acid oxidation and gluconeogenesis (Fi**gure 3-4A**).


A
Stored Triglycerides
Lipolysis
Cytoplasm
Mitochondria
scf000567_33894
β-oxidation
Gluconeogenesis
FOXO1
Adipogenesis
scf001086_36659
miR-149-3p
Fatty acid biosynthesis
PRKAG1
PGC-1a
Insulin signaling
CRTC1
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Extracellular Space
Extrasynaptic NMDAR
Cytoplasm
Uncontrolled Ca2+ influx
Excitotoxicity
Microtubule stability
Nucleus
Caspase activation
miR-4696
MAP6
PLEC
scf000152_19859
Caspase-3 cleavage
PARP1
PARP-1
scf000152_19859
CaMKII Activation
CAMKIIa
CaMKII
Apoptosis

Figure 3-4. Summary of biological processes under regulation of miRNAs during central bearded dragon hibernation. a Cellular metabolic processes common to all tissues assessed. **b** Cellular processes involved in the progression of excitotoxicity in neurons of brain. miRNAs with green arrows are upregulated during hibernation, with their target mRNAs downregulated. miRNAs shown with red arrows are downregulated during hibernation, with their target mRNAs upregulated. Red crosses refer to active regulation of the process during hibernation.

3.2.4.2 miRNAs facilitate neuroprotective mechanisms in the brain during hibernation

Neuronal microtubules are vital for proper neurotransmission. Specifically, microtubules and the microtubule-associated protein tau (MAPT) are essential for anchoring *N*-methyl-methyl-D-aspartate receptors (NMDARs) to the cell membrane; which are crucial to the control of synaptic plasticity and memory [288]. However, during cerebral stress, synaptic and extrasynaptic NMDARs are overactivated by excessive glutamate release. This causes uncontrolled intake of calcium ions (Ca^{2+}) and major neurological damage via induction of excitotoxicity [67]. In the brain of hibernating bearded dragons the NMDAR NR1 subunit *GRIN1* is downregulated, in addition to upregulation of tau protein kinases [268]. Increased phosphorylation of tau protein reduces its affinity for microtubules (reviewed in [289]), destabilizing the microtubule, and potentially causing disruption of NMDAR anchoring. Reduction of NMDAR anchoring to microtubules has been proposed to be a neuroprotective mechanism in hibernating mammals [71].

A novel bearded dragon miRNA (scf000152_19859) may be particularly important for regulating this process in hibernating individuals (**Figure 3-4B**). scf000152_1985, which was upregulated in the brain during hibernation, was predicted to target transcripts of the microtubule-associated proteins MAP6 and plectin (PLEC). Accordingly, mRNA and protein levels of MAP6, and protein levels of PLEC, were downregulated (**S3-3 Table**) [268]. MAP6 was shown to stabilise microtubules at cold temperatures (below 20°C), where its absence causes rapid depolymerisation of microtubules in HeLa and mouse embryonic fibroblast cells [277]. The two-fold reduction in MAP6 expression may result in microtubule depolymerisation in order to disrupt anchoring NMDARs in the brain during hibernation. PLEC is a very large protein that links actin, microtubules and intermediate filaments together [278]. Reduced abundance of plectin, with the addition of MAP6, may result in the further destabilisation of microtubules (**Figure 3-4B**).

CAMK2A was also downregulated during hibernation [268] and is another predicted target of scf000152_19859. *CAMK2A* encodes CaMKIIα, the neuronal-specific isoform of CaMKII, which is important in NMDAR signalling (reviewed in [290]). In excitotoxic neurons, CaMKII is persistently activated and initiates progression of apoptosis [291, 292]. Inhibition of CaMKIIα activity both prior to and after excitotoxic insult is extremely neuroprotective to rat neuronal cultures, with overexpression significantly increasing neuronal death [293, 294]. Our results suggest that the downregulation of CaMKIIα via miRNA activity may be vital for prevention of apoptosis progression in the brain of hibernation bearded dragons. Interestingly, in the tissues examined here scf000152_19859 transcripts were only detected in hibernating individuals, suggesting that scf000152_19859 may be a hibernation-specific miRNA.

PARP1 is a DNA damage sensor and repairs single-stranded DNA break. However, when PARP1 is cleaved by caspase-3, DNA damage repair is halted and apoptosis progresses [295, 296]. This process occurs in many neurological diseases, such as Parkinson's, Alzheimer's, cerebral ischemia (reduced blow flow) and NMDA-mediated excitotoxicity (reviewed in [295]). Upregulation of mir-4696, which is predicted to target *PARP1*, may downregulate *PARP1* during hibernation (**Figure 3-4B**). In mouse keratinocytes, downregulation of *PARP1* was associated with protection from radiation-induced apoptosis [297]. Therefore, downregulation of *PARP1* in brain during hibernation may reduce the potential for PARP1 cleavage and, hence, prevent progression of apoptosis.

These observations suggest miRNA-mediated gene expression helps protect against excitotoxicity in the brains of hibernating bearded dragons. Furthermore, changes in microtubule dynamics appear to mirror tauopathies (neurodegenerative disorders associated with aggregation of abnormal tau protein), including Alzheimer's and Parkinson's disease [298, 299]. The similar physiology in tauopathies and hibernation may suggest a common theme. In both cases, destabilization and reduced NMDAR signalling may be a neuroprotective process that reduces the potential for excitotoxicity. As such, much like mammalian hibernators [72, 300], the hibernating brain of bearded dragons could possibly be used as a model for neurological diseases, such as Alzheimer's and Parkinson's.

3.2.4.3 miRNAs target key translational repression genes

miRNA-mediated translational repression is thought to be vital to regulating the expression of proteins during hibernation [140, 301]. In bearded dragon, *AGO3* (the catalytic subunit of non-cleaving RISC), three key CCR4-NOT genes (necessary for

RISC-mediated translational repression) and *EIF4ENIF1* (a critical initiation factor for CCR4-NOT) were upregulated during hibernation [268]. Here we observed that four miRNAs targeting *AGO3*, *CNOT1* and *CNOT11* were each downregulated during hibernation (**Table 3-1, S3-3 Table**). This suggests that miRNAs may self-regulate the key genes involved in miRNA-mediated translational repression.

3.2.5 Conclusion

This study is the first small RNA profiling analysis of a reptile during hibernation and post-arousal. We identified conserved and novel miRNAs in the bearded dragon genome. Differentially expressed miRNAs that target key genes involved in cellular metabolism were uncovered, suggesting that miRNAs play a central role in regulating the phenotype of hibernating bearded dragons. Furthermore, the tissue-specific expression of miRNAs in the brain implies a role in regulating the expression of genes important for neuroprotection. Overall, this study reinforces the importance of miRNAs in regulating adaptive phenotypes, such as hibernation, and elucidates mechanisms that may be vital for survival during hibernation in the central bearded dragon.

3.2.6 Methods

3.2.6.1 Animals and tissue collection

Central bearded dragons (*Pogona vitticeps*) were captive bred and housed at the University of Canberra under a protocol approved by the University of Canberra Animal Ethics Committee (CEAE17-08) and ACT Government License to Keep (K9640). Husbandry practices fulfil the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) sections 3.2.13 - 3.2.23.

Captive conditions are as described in Capraro *et. al* (2019) [268]. All hibernation individuals are matched with that in Capraro *et. al* (2019), while the post-arousal individuals are not. Commercial sources of vegetables, mice and live insects (crickets and cockroaches) were provided as food, with water available *ad libitum*. Cages were cleaned thoroughly monthly, with superficial cleaning done daily (removal of faecal matter and unused food, maintenance of clean water containers). Logs and small branches were provided as basking perches and cardboard boxes provided as retreats. Enclosures were lit by a fluorescent lamp, a strong UVB light source, and a floodlamp (as a heat source) on a variable light:dark (L:D) cycle: August – mid-June (13hL:11hD; 22°C), late June (2 weeks- 6hL : 18hD; 18°C) and winter hibernation (0hL:24hD; 12°C). For two weeks prior to hibernation, heat and light were reduced and animals were not fed. All heat and UV lights were turned off for 8 weeks and the facility temperature maintained at 12°C, which stimulated any animals remaining active to hibernate. The conditions of artificial hibernation are chosen to mimic those that occur during natural hibernation, in that ambient temperatures are dropped, and light availability reduced. Body temperatures of hibernating animals was the same as ambient temperature (12°C) due to the lack of access to heat sources. After arousal from hibernation, animals were subject to full summer

conditions (13hL:11hD; 22°C). Body temperatures of animals was at least 22°C (ambient) with the addition of access to a heat source.

Whole brain, whole heart and femoral skeletal muscle tissue were collected from three individuals at two time points: late hibernation, and four days post-arousal (**Figure 3-5**). All lizards were male. Tissues were collected immediately after euthanizing (lethal injection of sodium pentobarbitone 65mg/kg by caudal venepuncture), snap frozen in liquid nitrogen and stored at -80°C until small RNA extraction. All post-arousal animals were sacrificed between zeitgeber time (ZT) 3 and ZT5, where ZT0 is lights on and ZT13 is lights off. Hibernating animals were sacrificed between circadian time (CT) 3 and CT5, where CT0 is the same time of day as ZT0, however, without lights turning on.

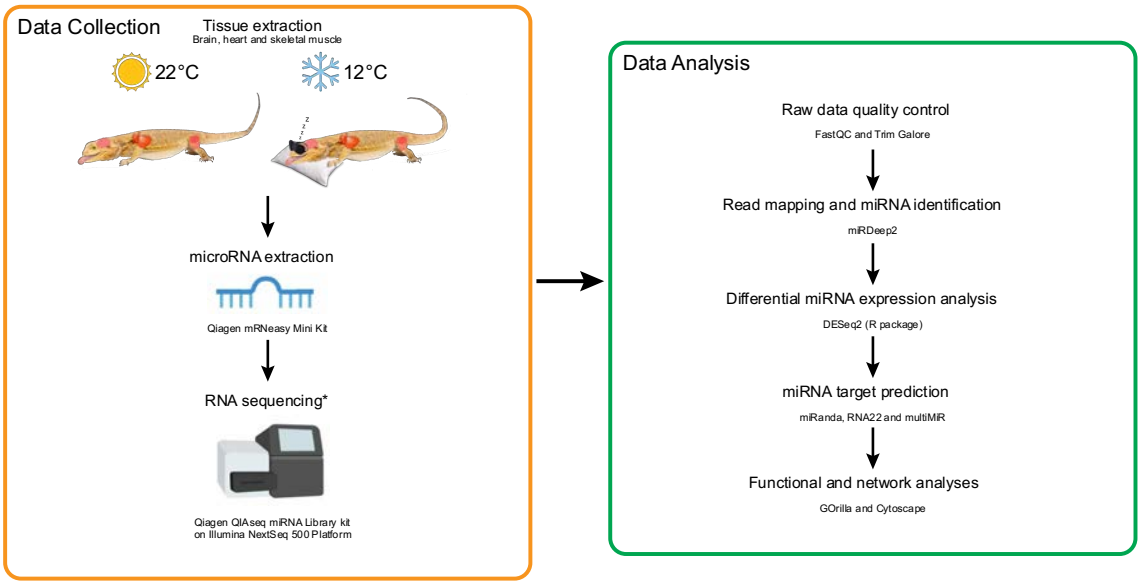


Figure 3-5. Experimental methods used to gather and analyse small RNA data. * Experimental protocol performed by the Ramaciotti Centre for Genomics, UNSW.

3.2.6.2 Small RNA Preparation and Sequencing

Total RNA was extracted from 30 mg of each tissue. Tissue extracts were homogenized using T10 Basic ULTRA-TURRAX® Homogenizer (IKA, Staufen im Breisgau, Germany), and RNA purified using the miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNase-free DNase (QIAGEN, Hilden, Germany) was used to digest DNase on-column. For each sample, 500 ng of high integrity total RNA (RIN > 9) was used for sequencing library construction with the QIAseq miRNA Library Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Seventy-five bp single-ended reads were generated on the Illumina NextSeq 500 platform at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia). All sequence data have been submitted to the NCBI sequence read archive under the BioProject ID PRJNA605672.

3.2.6.3 Bioinformatics Analysis

Raw sequencing reads were analysed with FastQC (v0.11.5) [256] and low quality bases were removed using Trim Galore (v0.0.4.4) [302] with the following options: --phred33 –gzip –length 16 --max_length 24 –adapter AACTGTAGGCACCATCAAT --three_prime_clip_R1 1. miRDeep2 (v2.0.1.2) [258] was used to map reads to the genome, predict conserved and novel miRNAs, and quantify number of miRNA reads using the following settings: -d -e -h -i -j -l 18 -p -v -n --o 8. Predicted miRNAs were compared to the miRBase databases (Release 22.1) [303] of known human (*Homo sapiens*) and green anole (*Anolis carolinensis*) miRNAs. miRNAs with a miRDeep2 score of greater than 4 were considered real; conferring to a signal-to-noise ratio of greater than 8.1:1. Differential expression analysis of miRNAs was performed with DESeq2 (v1.22.2) [304].

All graphs were plotted with R (3.4.2) [263], RStudio (1.1.383) [264], and ggplot2 (2.2.1) [265]. miRNAs with a $\log_2$ fold-change greater than 0.75 and adjusted p-value less than 0.05 were considered differentially expressed. miRanda (v3.3a; with the options: -sc 150 -en -20 -strict) [274] and RNA22 (v2.0; with default options) [275] were used to predict the target mRNAs of the DEmiRs (**Figure 3-1D, S3-2 Table**). For conserved miRNAs, targets were predicted by multiMiR (v1.4.0) [276].

mRNA-seq and proteomic data was gathered from Capraro *et. al* (2019) [268]. Gene ontology (GO) enrichment analysis was performed with GOrilla on differentially expressed target mRNAs using mRNA-seq data (last accessed 28/8/19) [261]. Unranked lists of upregulated and downregulated genes in each condition and tissues were compared to a background list; genes that were expressed (greater than 10 counts per million) within each tissue. Network analysis of differentially expressed miRNAs and their target mRNAs was performed using Cytoscape (v3.7.1) [279]. All graphs were plotted with R (3.4.2) [263], RStudio (1.1.383) [264], and ggplot2 (2.2.1) [265].

3.2.7 Declarations

3.2.7.1 Ethics approval and consent to participate

Experimentation using animals was approved by the University of Canberra Animal Ethics Committee (CEAE17-08) and are in accordance with ACT Government License to Keep (K9640). Husbandry practices fulfil the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) sections 3.2.13 - 3.2.23.

3.2.7.2 Availability of data and materials

RNA-seq data are available in the NCBI sequence read archive under the BioProject ID PRJNA476034 (http://www.ncbi.nlm.nih.gov/bioproject/476034) and miRNA-seq data is available under the BioProject ID PRJNA605672 (https://www.ncbi.nlm.nih.gov/bioproject/605672). Computer code for processing and analysing sequence and mass spectrometry data is available on request.

3.2.7.3 Competing interests

The authors declare that they have no competing interests.

3.2.7.4 Consent for publication

Not applicable.

3.2.7.5 Funding

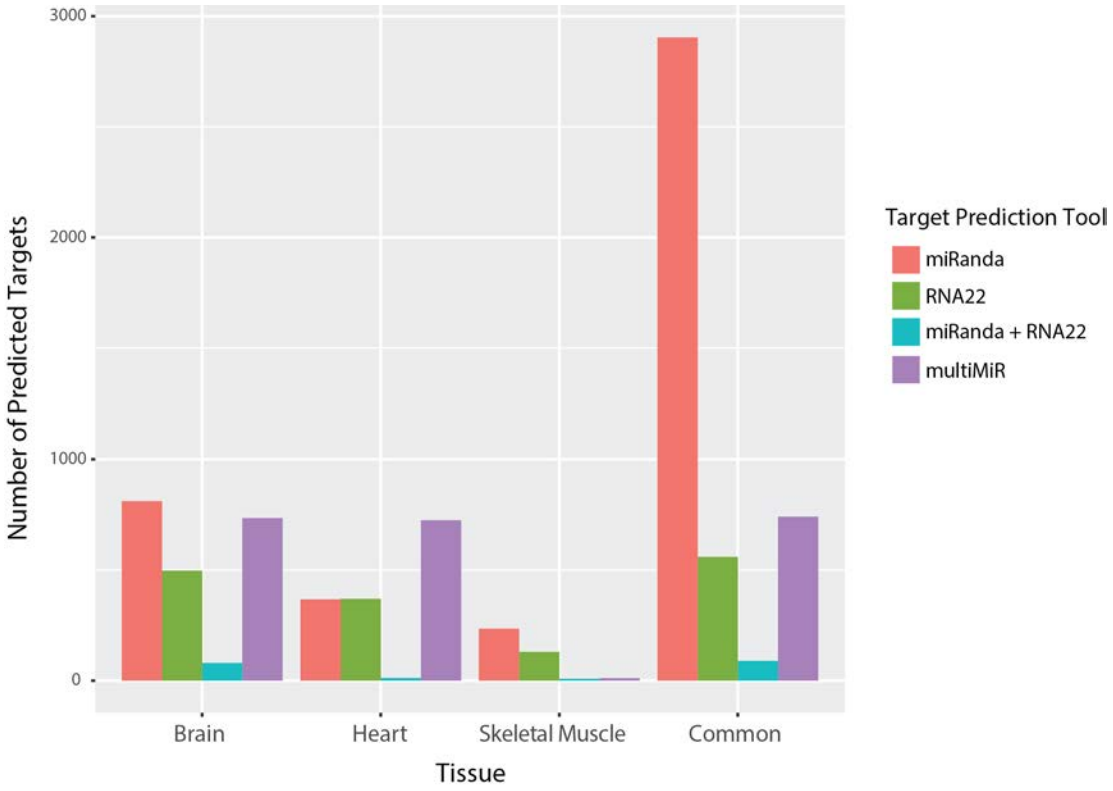
The project was funded by internally allocated funds from UNSW Sydney and in part by a grant from the Australian Research Council (DP170101147) awarded to AG and PW. The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

3.2.7.6 Authors' contributions

PDW, AC, DO, SW and HP conceived and designed the study. AC performed the experiments. AC performed the computational analysis of sequencing data and statistical analyses. AG provided tissue samples. AC and PDW wrote the paper. All authors read and approved the final manuscript.

3.2.8 Appendices

3.2.8.1 Supplementary Figures



S3-1 Figure. Number of predicted mRNA targets of differentially expressed microRNAs. Total number of mRNA targets of all differentially expressed miRNAs predicted by miRanda (red), RNA22 (green), both miRanda and RNA22 (blue), and multiMiR (purple).

3.2.8.2 Supplementary Tables

Supplementary files are not included in this chapter due to excessive length and can be accessed via the following file sharing link: https://www.dropbox.com/sh/wf77mb23plipt0l/AACCXxTvk6ORSufo2tfpIdZoa?dl=0.

Supplementary table legends are provided below.

Additional File 1: S3-1 Table. Annotation of microRNAs in the central bearded dragon genome. Full list of all annotated microRNAs in the central bearded dragon genome as outputted from miRDeep2.

Additional File 3: S3-2 Table. mRNA targets of all differentially expressed microRNAs. Full list of mRNA targets of all differentially expressed microRNAs. The list contains mRNA targets of conserved miRNAs predicted by multiMiR and targets of novel miRNAs predicted by miRanda and RNA22.

Additional File 4: S3-3 Table. Differential gene expression of mRNA targets of differentially expressed microRNAs. Full list of mRNA target expression of differentially expressed miRNAs for brain, heart, skeletal muscle, and common genes as outputted from EdgeR. Differentially expressed genes were gathered from Capraro *et al*. (2019). Log_2 fold change is relative to hibernation (*i.e.* > 1 Log_2FC is higher expression during hibernation).

Additional File 5: S3-4 Table. Gene ontology enrichment analysis data of differentially expressed mRNA targets of differentially expressed microRNAs. Full list of enriched biological pathway gene ontologies in upregulated and downregulated mRNA target datasets in brain, heart, skeletal muscle, and common genes as outputted from GOrilla.

4 DNA methylation changes in hibernation

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4.1 DNA methylation changes result in adaptive responses in the hibernating central bearded dragon

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Current status of paper:

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Extent to which research is your own:

I performed all the experiments and all analyses for this manuscript. These include RNA extraction, DNA extraction, and bioinformatics and statistical analysis of all data.

Your contribution to writing the paper:

I wrote all manuscript drafts and generated all figures.

If paper has not yet been accepted, has the paper been rejected by any journals:

No.

Comments:

The following manuscript is still in the drafting stage as it requires input and drafting by co-authors. Supplementary tables are not included in this chapter due to excessive length and can be accessed via the following link: https://www.dropbox.com/sh/j11fj1wgt8414xb/AAAP4g1wcbPVf46PR4A90ZNOa?dl=0.

4.2 Draft manuscript

DNA methylation changes result in adaptive responses in the hibernating central bearded dragon

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4.2.1 Abstract

Hibernation is a seasonal physiological adaptive response used by diverse animal species to survive months of starvation and stress during winter. It involves large changes in cell, tissue and organ physiology to maintain function and reduce energy expenditure. In order to implement and maintain these adaptive responses, hibernators employ diverse gene regulatory mechanisms, including DNA methylation. CpG methylation patterns, particularly at the transcription start site and promoter regions of genes, are known to correlate with gene expression. However, studies investigating the role of DNA methylation in hibernators are lacking, limited to only specific genes and in non-specific cytosine contexts. For the first time, using reduced representation bisulfite sequencing, we profiled CpG methylation in the brain and skeletal muscle of the Australian central bearded dragon (*Pogona vitticeps*) during late hibernation, two days post-arousal and two months post-arousal. During hibernation differential DNA methylation correlated with differential expression of key neuroprotective genes in the brain, and atrophy prevention genes in skeletal muscle. These data provide the first gene specific evidence that DNA methylation plays a role in regulating stress response and preservation pathways in a hibernating reptile.

4.2.2 Background

Hibernation is a seasonal physiological response to fluctuations in environmental temperatures, photoperiods, and food availability. A diverse range of animals utilize hibernation from reptiles, birds, monotremes (echidna), rodents and primates (dwarf-lemur) [1, 2]. While the precise alterations in physiology and behaviour vary from species to species, hibernation characteristically occurs during the winter months where periods of prolonged dormancy and metabolic depression (torpor) are temporarily interrupted by short periods of arousal (interbout arousal) [163]. The long intervals of torpor require organism-wide alterations in fundamental biological processes. Reduced basal metabolic rate, transcriptional and translational rates, oxygen consumption, heart rate and core body temperatures are among the most drastic changes [3]. A common strategy shared among hibernators is the reprioritisation of fuel sources from the regular utilization of glucose-based energy sources, to stored fatty-acid and lipid-based sources [3].

Induction of hibernation subjects the animals to varied stresses, including cold stress, hypoxia, ischemia-reperfusion injury (damage caused by reduced blood flow followed by subsequent resupply of blood), nutrient deficiency, and a compromised immune system. This necessitates the need for global and tissues-specific responses to thwart otherwise fatal conditions. Two organs particularly vulnerable to this insult are the brain and skeletal muscle.

In the brain, oxygen and glucose deprivation leads to depolarisation of the plasma membrane, resulting in increased release of glutamate into the extracellular space [63]. Increased extracellular glutamate causes excitotoxicity of neurons; a process whereby excitatory neurotransmitters such as the *N*-methyl-D-aspartate receptor (NMDAR) are

overactivated leading to excess of calcium ion release and ultimately activating apoptotic pathways [64-67]. Hibernating squirrels prevent excitotoxicity progression by downregulating NMDAR function to reduce Ca^{2+} influx [68, 69].

Skeletal muscle of hibernating animals is expected to suffer from large reductions in muscle cell count, size, and contractile strength due to the long periods of inactivity. However, hibernating animals display the remarkable ability to prevent skeletal muscle atrophy that non-hibernators, such as humans, suffer in as little as five days [93, 305]. One such mechanism to prevent disuse atrophy is shifting from fast-twitch type II muscle fibres to slow-twitch type I muscle fibres [94-96]. The switch in muscle fibre type is thought to promote proper maintenance of muscle homeostasis and fatigue-resistant muscle [96, 306].

These complex alterations are mediated by a multi-level gene expression regulatory system. Evidence for transcriptional, post-transcriptional, translational, and post-translational regulation of the hibernation phenotype exists (reviewed in [307]). A particularly interesting regulatory mechanism is DNA methylation. In vertebrates, DNA methylation is a fundamental epigenetic mechanism that involves the covalent addition of methyl groups (CH_3) to carbon-5 position of cytosine to form 5-methylcytosine (5mC) by DNA methyltransferases (DNMTs).

DNA methylation is important in the context of CpG dinucleotides where it is involved in a vast number of key biological processes, including cellular differentiation, tissue-specific gene expression, aging, cancer, and X-chromosome inactivation. DNA methylation acts to physically modulate the access of transcriptional machinery and

transcription factors to DNA [114]. Methylated DNA also binds methyl-CpG-binding domain proteins (MBDs) which recruit histone modifying proteins [115].

Due to the apparent roles of DNA methylation in key biological processes, there is interest in the role that DNA methylation plays in hibernation. In hibernating thirteen-lined ground squirrel global DNA methylation increases in brown adipose tissue [103], decreases in skeletal muscle, and remains constant in liver [104]. This suggests that global DNA methylation levels may have a tissue-specific role in mammalian hibernation. However, to date only three studies have investigated DNA methylation in hibernators, all of which are restricted to mammals [103, 104, 120]. These studies focused on global cytosine methylation, and methylation of one specific gene promoter. There is a lack of high-throughput molecular investigations in methylation states of non-mammalian and mammalian hibernators alike.

The Australian central bearded dragon (*Pogona vitticeps*) is an excellent model to study the dynamics of DNA methylation in reptilian hibernation due to the ease of hibernation induction, sequenced genome [166], and ability to correlate with previous RNA-sequencing data in matched tissues [268]. Here we profiled DNA methylation using RRBS in brain and skeletal muscle at three time points: 1) late hibernation; 2) two days post-arousal (pre-feed); and 3) two months post-arousal. Differentially methylated regions of gene promoters and gene bodies were identified and correlated with previously published RNA-seq data of matched tissue. We identified key differentially methylated genes involved in neuroprotection in the brain, and prevention of muscle atrophy in skeletal muscle.

4.2.3 Results

In brain and skeletal muscle of central bearded dragons reduced representation bisulfite sequencing (RRBS) was used to measure DNA methylation at CpG sites. Individuals were sampled at late hibernation, two days post-arousal and two months post-arousal. Methylation profiles for each tissue at each time point permitted assessment of global DNA methylation, and DNA methylation at meta-genomic features. Furthermore, we analysed differentially methylated regions (DMRs), promoters and gene bodies to identify specific genes that were potentially regulated by DNA methylation during hibernation. Finally, we analysed enriched biological pathways of differentially methylated genes to determine the pathways under DNA methylation-dependent transcriptional control between the hibernating and aroused states.

4.2.3.1 Global and meta-genomic feature CpG methylation levels

Across both tissues and all time points, there was a bimodal distribution of DNA methylation levels, where CpGs were either hypermethylated (>90% methylated) or hypomethylated (<10% methylated; **Figure 4-1**). In brain, DNA methylation was highest two days post-arousal (mean: 46.46%), and lowest during hibernation (mean: 45.44%; **Figure 4-1A**). Conversely, in skeletal muscle DNA methylation was highest during hibernation (mean 44.04%), and lowest two months post-arousal (mean 43.63%). In brain, differences between time points were all statistically significant according to a student's *t*-test ($p < 1.92 \times 10^{-6}$). In skeletal muscle comparison of all time points apart from two days post-arousal and two months post-arousal ($p = 0.277$) were statistically significant ($p < 1.92 \times 10^{-6}$). Although statistically significant, the change in global CpG

methylation between time points was small, with a maximum change of 1.01% observed between hibernation and two days post-arousal in brain.

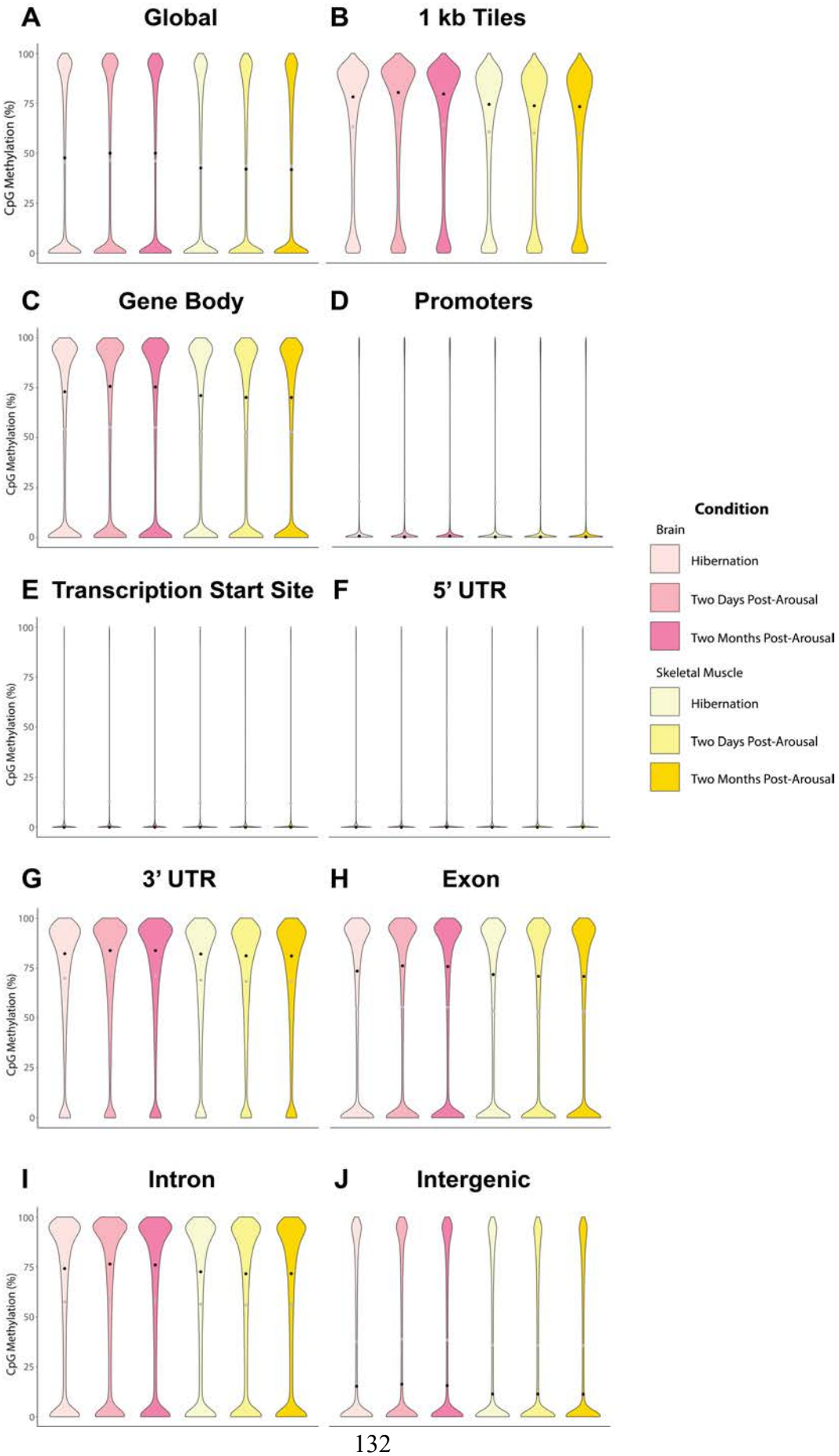
A
Global
B
1 kb Tiles
C
Gene Body
D
Promoters
E
Transcription Start Site
F
5' UTR
G
3' UTR
H
Exon
I
Intron
J
Intergenic
CpG Methylation (%)
100
75
50
25
0
Condition
Brain
Hibernation
Two Days Post-Arousal
Two Months Post-Arousal
Skeletal Muscle
Hibernation
Two Days Post-Arousal
Two Months Post-Arousal

Figure 4-1. Metagenomic distribution of CpG methylation. A Global. **B** 1 kb tiles covering the entire genome. **C** Gene bodies. **D** Promoters (-2 kb and +200 bp from TSS). **E** Transcription start site. **F** 5' untranslated region. **G** 3' untranslated **H** Exons. **I** Introns. **J** Intergenic regions. Black and grey dots show median and mean CpG methylation, respectively.

We performed a meta-analysis of CpG methylation within annotated genomic features, including: 1 kb tiles across the genome (**Figure 4-1B**), gene bodies (**Figure 4-1C**), promoters (**Figure 4-1D**), transcription start sites (TSSs; **Figure 4-1E**), 5' UTR (**Figure 4-1F**), 3' UTR (**Figure 4-1G**), exons (**Figure 4-1H**), introns (**Figure 4-1I**), and intergenic regions (**Figure 4-1J**). CpG methylation was highest in 3' UTRs (85–89%), followed by 1kb tiles (75–80%) and exons (70–76%). The lowest CpG methylation was observed at the TSSs and in 5' UTRs (1%). These patterns were reflected in both brain and skeletal muscle for the three time points. Much like the global CpG methylation, these differences between time points were small.

4.2.3.2 Chromosome level CpG methylation

CpG methylation across chromosomes was assessed to identify regions with changes in methylation between hibernation and post-arousal time points. The central bearded dragon genome is only assembled into scaffolds. Therefore, multiple adjacent scaffolds anchored to chromosomes by Deakin et al. (2016) [167] (called superscaffolds) were used for these analyses. As such, known regions of chromosomes 1, 2, 3, 4, 6, 11 and the Z were assessed (**Figure 4-2**).

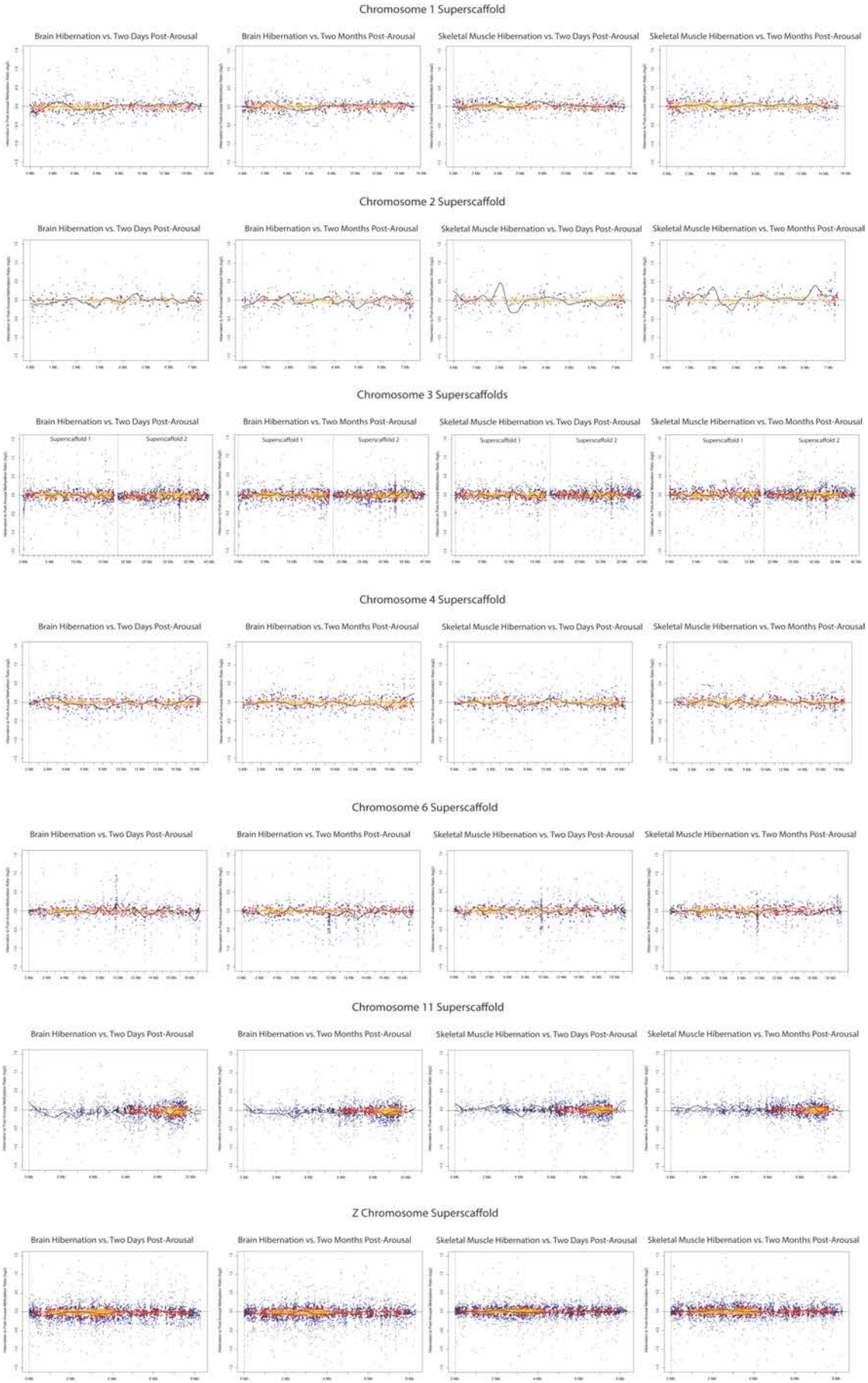
Chromosome 1 Superscaffold
Brain Hibernation vs. Two Days Post-Arousal
Brain Hibernation vs. Two Months Post-Arousal
Skeletal Muscle Hibernation vs. Two Days Post-Arousal
Skeletal Muscle Hibernation vs. Two Months Post-Arousal
Chromosome 2 Superscaffold
Brain Hibernation vs. Two Days Post-Arousal
Brain Hibernation vs. Two Months Post-Arousal
Skeletal Muscle Hibernation vs. Two Days Post-Arousal
Skeletal Muscle Hibernation vs. Two Months Post-Arousal
Chromosome 3 Superscaffolds
Brain Hibernation vs. Two Days Post-Arousal
Brain Hibernation vs. Two Months Post-Arousal
Skeletal Muscle Hibernation vs. Two Days Post-Arousal
Skeletal Muscle Hibernation vs. Two Months Post-Arousal
Superscaffold 1
Superscaffold 2
Chromosome 4 Superscaffold
Brain Hibernation vs. Two Days Post-Arousal
Brain Hibernation vs. Two Months Post-Arousal
Skeletal Muscle Hibernation vs. Two Days Post-Arousal
Skeletal Muscle Hibernation vs. Two Months Post-Arousal
Chromosome 6 Superscaffold
Brain Hibernation vs. Two Days Post-Arousal
Brain Hibernation vs. Two Months Post-Arousal
Skeletal Muscle Hibernation vs. Two Days Post-Arousal
Skeletal Muscle Hibernation vs. Two Months Post-Arousal
Chromosome 11 Superscaffold
Brain Hibernation vs. Two Days Post-Arousal
Brain Hibernation vs. Two Months Post-Arousal
Skeletal Muscle Hibernation vs. Two Days Post-Arousal
Skeletal Muscle Hibernation vs. Two Months Post-Arousal
Z Chromosome Superscaffold
Brain Hibernation vs. Two Days Post-Arousal
Brain Hibernation vs. Two Months Post-Arousal
Skeletal Muscle Hibernation vs. Two Days Post-Arousal
Skeletal Muscle Hibernation vs. Two Months Post-Arousal

Figure 4-2. **CpG methylation patterns across chromosome superscaffolds.** Heatscatter plots of hibernation to post-arousal time points CpG methylation ratios across the length of superscaffolds of known regions of chromosomes 1, 2, 3, 4, 6, 11 and the Z. Each dot represents the hibernation to post-arousal ratio within a 10 kb window on a $\log_2$ scale. Above zero is higher methylation during hibernation, below zero is higher methylation post-arousal. Colour represents data density, with yellow representing high and grey representing low. Smoothed lines of best fit are black. See https://www.dropbox.com/sh/j11fj1wgt8414xb/AAAP4g1wcbPVf46PR4A90ZNOa?dl=0 for full resolution image.

CpG methylation on chromosomes during hibernation was compared to both two days post-arousal and two months post-arousal in brain and skeletal muscle. Generally, CpG methylation was consistent across all chromosomes between hibernation and post-arousal, with specific regions of differential methylation.

On the chromosome 1 superscaffold, at ~2.3 Mb DNA methylation was ~10% higher during hibernation than after arousal from hibernation in brain (**Figure 4-2**). Within this region was the gene *CD6*. On superscaffold 1 of chromosome 3, the largest difference in DNA methylation of any chromosome occurred at the start of the scaffold in brain between 0 Mb and 1 Mb (**Figure 4-2**). During hibernation, methylation was 35% lower than two months post-arousal and 30% lower than two days post-arousal. This region bears the gene *EMX2*; which encodes a homeobox protein important in the central nervous system [308].

On the chromosome 6 superscaffold, a region of high methylation variability was observed at 9.6 Mb, which overlaps the *HOXA* cluster (**Figure 4-2**). In brain, during hibernation DNA methylation was 15% higher compared to two days post-arousal but compared to two months post-arousal DNA methylation was 5% lower. This reduced methylation during hibernation was also observed in skeletal muscle.

On the chromosome 11 superscaffold, changes in DNA methylation between hibernation and post-arousal time points was tissue-specific (**Figure 4-2**). CpG sites were more methylated during hibernation in brain, which contrasted skeletal muscle where CpG sites were more methylated post-arousal. For example, from 0.5 Mb to 3 Mb, DNA methylation was reduced (~5% - 15%) in brain, while methylation was increased (~5% - 10%) in skeletal muscle. This region contains the neuronal genes *NTM* and *OPCML* (1.6 Mb to 2.2 Mb). Additionally, from 6 Mb to 9 Mb, there were several cell adhesin molecules (*NECTIN1*, *NCAM1* and *CADM1*) and a dopamine receptor (*DRD2*). This region had increased methylation during hibernation in brain, but not in skeletal muscle.

4.2.3.3 CpG methylation at transcription start sites

CpG methylation at transcription start sites is a critical regulatory mechanism in vertebrates [309]. We correlated CpG methylation profiles at TSSs, and mRNA expression of matched tissue [268]. Across all tissues and time points, we observed an inverse relationship between TSS methylation level and mRNA expression (**Figure 4-3**). Genes with low expression have a flat methylation profile across the promoter and in the gene body. As the expression of genes increased, TSS methylation decreased and gene body (~2.5 kb upstream) methylation increased.

In brain, promoter methylation (~2.5 kb upstream) decreased as expression increased. However, in skeletal muscle, upstream methylation remained relatively constant (**Figure 4-3)**. This pattern of TSS methylation was consistent at all time points. TSS methylation of genes with low expression (expression octiles 1 – 3) in brain was drastically higher than in skeletal muscle, but highly expressed genes (expression octiles 6 – 8) were comparable (**Figure 4-3**). Interestingly, in skeletal muscle, the methylation levels of TSSs of genes in expression octile 8 (>50 TPM) was higher than those in expression octile 7 (30 – 50 TPM; **Figure 4-3D- F**). However, the difference between these octiles was small (0.1% and 1.3% respectively), and not significant (student's *t*-test, p = 0.283 and 0.537, respectively).

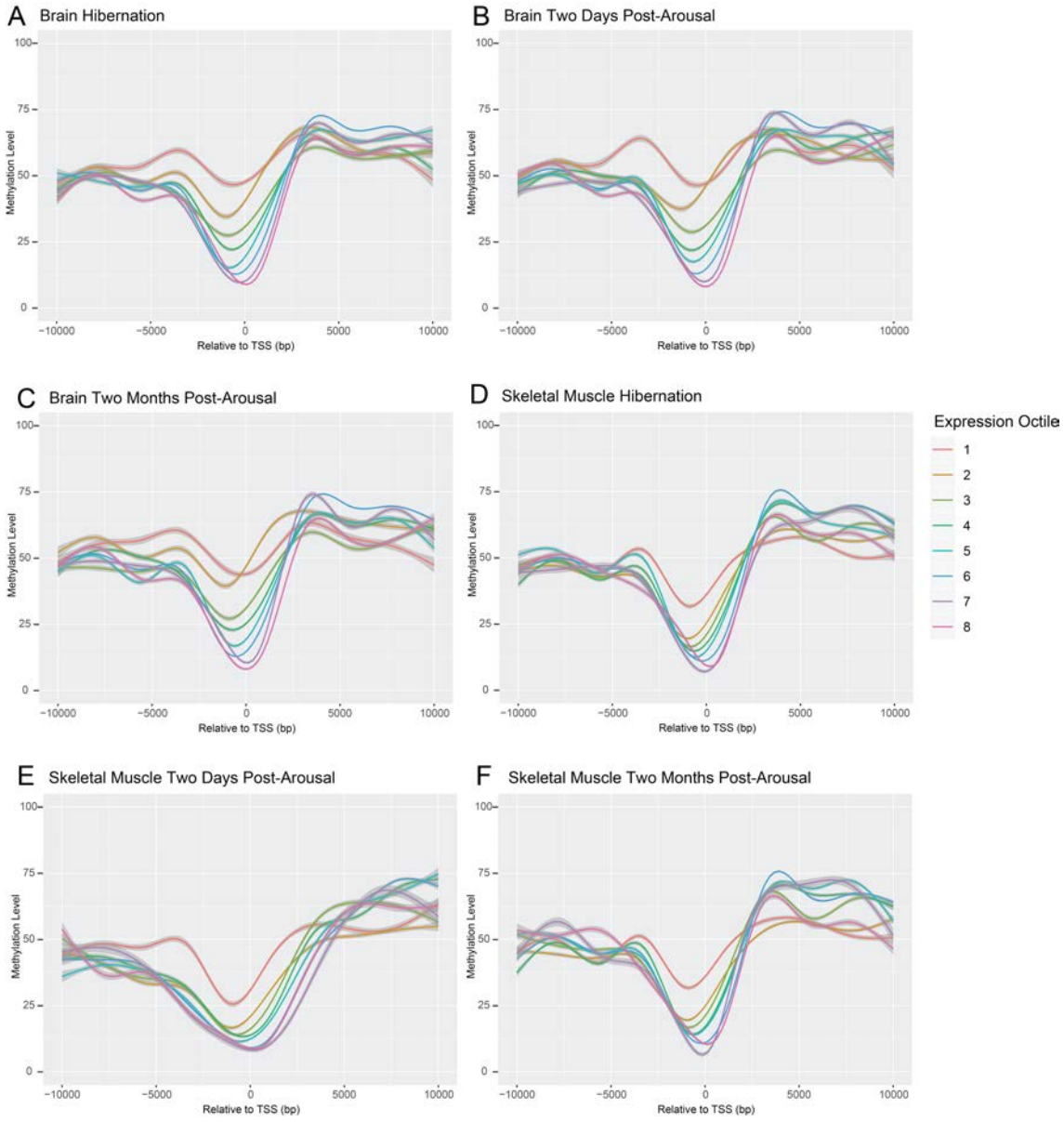

Figure 4-3. Relationship between transcription start site methylation and gene expression. Smoothed lines of the mean CpG methylation of all annotated genes ±10 kb the transcription start site in brain (**A – C**) and skeletal muscle (**D – F**). Genes are classified into expression octiles (8 = highest) according to expression levels in matched tissue RNA-seq data (data from Capraro *et al.* 2019). Grey shadowing are 95% confidence intervals.

4.2.3.4 Differentially methylated promoters and gene bodies

We analysed significantly differentially methylated (differential methylation >20% and q-value < 0.05) promoters (-2 kb and +200 bp relative to annotated TSSs) of protein-coding genes. Three pairwise comparisons were performed for both tissues: 1) hibernation versus two days post-arousal (Hv2D); 2) hibernation versus two months post-arousal (Hv2M); and 3) two days post-arousal versus two months post-arousal (2Dv2M). The number of differentially methylated promoters was greater in brain than in skeletal muscle (**Table 4-1, S4-1 Table**). In brain, the largest number of significantly differentially methylated promoters (DMPs) was between Hv2D, followed by Hv2M then 2Dv2M (**Table 4-1, S4-1 Table**). In skeletal muscle, the greatest number of DMPs was between 2Dv2M, followed by Hv2M then Hv2D (**Table 4-1, S4-1 Table**). DMPs were correlated with previously published [268] differentially expressed genes (DEGs; $\log_2$ fold change > 0.565; false discovery rate < 0.05) of matched tissues (**Table 4-1, S4-3 Table**). Differential promoter methylation was better correlated with differential mRNA expression in brain than in skeletal muscle.

Table 4-1. Number of differentially methylated promoters (DMPs) and gene bodies (DMGs).

Time point comparison	DMPs (hyper/hypo)	Differentially expressed genes with DMPs (hyper/hypo)	DMGs (hyper/hypo)	Differentially expressed genes with DMGs (hyper/hypo)
Brain				
Hv2D	162 (125/37)	35 (24/11)	141 (109/32)	23 (20/3)
Hv2M	116 (86/30)	26 (16/10)	115 (72/43)	19 (14/5)
2Dv2M	71 (34/37)	0	97 (34/63)	0
Skeletal Muscle				
Hv2D	29 (16/13)	3 (2/1)	70 (33/37)	12 (7/5)
Hv2M	63 (23/40)	9 (5/4)	89 (35/54)	9 (5/4)
2Dv2M	97 (42/55)	0	69 (28/41)	0

Significantly differentially methylated gene bodies (DMGs) were also analysed. In brain, the comparison with the most differentially methylated gene bodies was Hv2D, followed by Hv2M and 2Dv2M (**Table 4-1, S4-1 Table**). For skeletal muscle, the comparison with the most differentially methylated gene bodies was Hv2M, followed by Hv2D and 2Dv2M (**Table 4-1, S4-1 Table**). DMGs were also correlated with DEGs (**Table 4-1, S4-3 Table**). As for DMPs, DMGs were more highly correlated with differential gene expression in brain than in skeletal muscle.

4.2.3.5 Differentially methylated gene bodies

Differentially methylated regions (DMRs; differential methylation >20% and q-value < 0.05) of varying lengths (~50 bp to > 500 bp) were calculated with the R package edmr (see **Chapter 4.2.6.4**). These were intersected with predicted promoters (-2 kb and +200 bp relative to the TSSs). Much like in the differential methylation analysis of promoters, the number of DMRs was considerably higher in brain than in skeletal muscle. In both tissues, Hv2D had the most DMRs within promoters, followed by Hv2M then 2Dv2M (**Table 4-2, S4-2 Table**). DMRs intersecting promoters were also correlated with DEGs (**Table 4-2, S4-4 Table**), and had a higher correlation than observed for DMPs.

Table 4-2. Number of differentially methylated regions (DMRs) that intersect with promoters and gene bodies.

Time point comparison	DMRs in promoters (hyper/hypo)	Differentially expressed genes with DMRs in promoters (hyper/hypo)	DMRs in gene bodies (hyper/hypo)	Differentially expressed genes with DMRs in gene bodies (hyper/hypo)
Brain				
Hv2D	68 (58/10)	17 (16/1)	514 (427/87)	123 (101/22)
Hv2M	34 (29/5)	4 (3/1)	359 (301/58)	90 (75/15)
2Dv2M	25 (17/8)	0	158 (59/90)	0
Skeletal Muscle				
Hv2D	13 (6/7)	3 (2/1)	108 (40/68)	35 (16/19)
Hv2M	11 (4/7)	4 (3/1)	139 (61/78)	39 (18/21)
2Dv2M	10 (6/4)	0	112 (54/58)	1 (0/1)

A larger number of DMRs intersected gene bodies compared to promoter regions. In brain, Hv2D comparisons yielded the largest number of DMRs followed by Hv2M, then 2Dv2M (**Table 4-2, Figure 4-4B, S4-2 Table**). In skeletal muscle, the largest number of DMRs that intersected gene bodies was Hv2M, followed by Hv2D, then 2Dv2M (**Table 4-2, Figure 4-4B, S4-2 Table**) DMRs that intersected gene bodies were also correlated with the gene expression (**Table 4-2, Figure 4-4D, S4-4 Table**). In general, DMRs in gene bodies were better correlated than differentially methylated gene bodies.

4.2.3.6 Functional analysis of differentially methylated genes

Gene ontology (GO) enrichment analyses were performed for all genes (promoter and gene body) that intersected a DMR (**Figure 4-5**). For the 582 genes with DMRs between hibernation and two days post-arousal in brain, 482 significantly enriched (FDR <0.05) biological process GO terms were identified (**S4-5 Table**). There was an enrichment of signalling pathways, including regulation of small GTPase mediated signal transduction (23 genes), Rho protein signal transduction (16 genes), and synaptic signalling (29 genes; **S4-5 Table**). Five key genes (*EPS8*, *ARHGEF11*, *ARHFEG18*, *DNMBP* and *MYO9B*) involved in Rho signal transduction that were differentially methylated were also differentially expressed. Furthermore, the *RHOA* gene body methylation increased 20% during hibernation compared to two days post-arousal (**S4-3 Table**) and was upregulated in brain during hibernation [268].

Genes involved in neuronal organization were differentially methylated in brain during hibernation compared to two days post-arousal, with biological process enrichments for regulation of cellular component organization (99 genes), cytoskeleton organization (49 genes), nervous system development (68 genes), NMDA glutamate receptor clustering (2 genes) and regulation of synapse assembly (11 genes; **S4-5 Table**). The two NMDA glutamate receptor associated genes (*NRXN1* and *SHANK3*) were not differentially expressed. However, two genes important in glutamate receptor signalling (*GRID2IP* and *GRIK3*) that had hypomethylation in the gene body were downregulated (**S4-4 Table**). Genes that intersected DMRs were also enriched for stress response pathway genes, including response to hypoxia (16 genes) and cellular response to glucose starvation (6 genes; **S4-5 Table**). Five of the hypoxia related genes were differentially expressed

during hibernation, with *ALKBH5, ANGPTL4* and *HSF1* upregulated, and *ERCC2* and *ALAD* downregulated.

For the 393 genes with DMRs between hibernation and two months post-arousal in brain, there were 500 significantly enriched biological process GO terms (**S4-5 Table**). These enrichments also included signalling pathways, including regulation of small GTPase mediated signal transduction (20 genes), synaptic signalling (29 genes), regulation of Ras protein signal transduction (11 genes), and regulation of Rho protein signal transduction (9 genes; **S4-5 Table**). Two genes were downregulated (*EPS8* and *DNMBP*) and one upregulated (*SCAI*) in the Rho signalling pathway. Neuron specific processes including synapse assembly (10 genes), synapse organization (19 genes), NMDA glutamate receptor clustering (2 genes) and regulation of NMDA receptor activity (3 genes) were also enriched (**S4-5 Table**). Two genes involved in synapse assembly *OXT* and *AGRN* were up- and downregulated, respectively.

Enrichment of stress response pathways was also observed, including cellular response to stress (42 genes) and response to hypoxia (11 genes; **S4-5 Table**). Seven genes involved in stress response were upregulated (*HSF1*, *NCOR1*, *RAPGEF1*, *MTMR3*, *MFHAS1*, *GAS2L1*, and *ALKBH5*) and three downregulated (*MAPKAPK5*, *RAD51C* and *DYSF*). The genes *ALKBH4* and *HSF1* (involved in hypoxia response) were also differentially methylated and upregulated during hibernation compared to two days post-arousal.

In skeletal muscle, the 121 genes that intersected DMRs (either promoter and/or gene body) between hibernation and two days post-arousal were significantly enriched for 71 biological process GO terms (**S4-5 Table**). A number of these processes were related to

muscle processes including actin cytoskeleton organization (11 genes), response to muscle inactivity (2 genes), and muscle atrophy (2 genes; **S4-5 Table**). Two of the actin cytoskeleton genes (*CGNL1*, and *TNXB*) were downregulated and one (*BCR*) was upregulated. Furthermore, the promoter of *ACTN3* and the gene body of *MYOZ2* (both actin cytoskeleton genes) were differentially methylated during hibernation (**S4-1 Table**). *MYOZ2* is downregulated during hibernation compared to two days post-arousal (**S4-3 Table**), while *ACTN3* was previously found to be downregulated during hibernation in bearded dragon [268]. Metabolic processes were also enriched, including cellular ketone metabolic process (7 genes), response to nutrient levels (8 genes), and response to insulin (6 genes; **S4-5 Table**).

The skeletal muscle hibernation and two months post-arousal analysis revealed 61 significantly enriched (FDR <0.05) biological process GO terms (**S4-5 Table**). These included postsynaptic specialization organization (4 genes), synaptic vesicle cycle (6 genes), and lipid metabolic processes (17 genes; **S4-5 Table**).

4.2.3.7 Transcription start site methylation of genes of interest

The CpG methylation profiles across the TSSs of genes with differential methylation and differential expression were explored (**Figure 4-4**). However, the TSS methylation pattern of some genes could not be established due to low sequence coverage and/or depth.

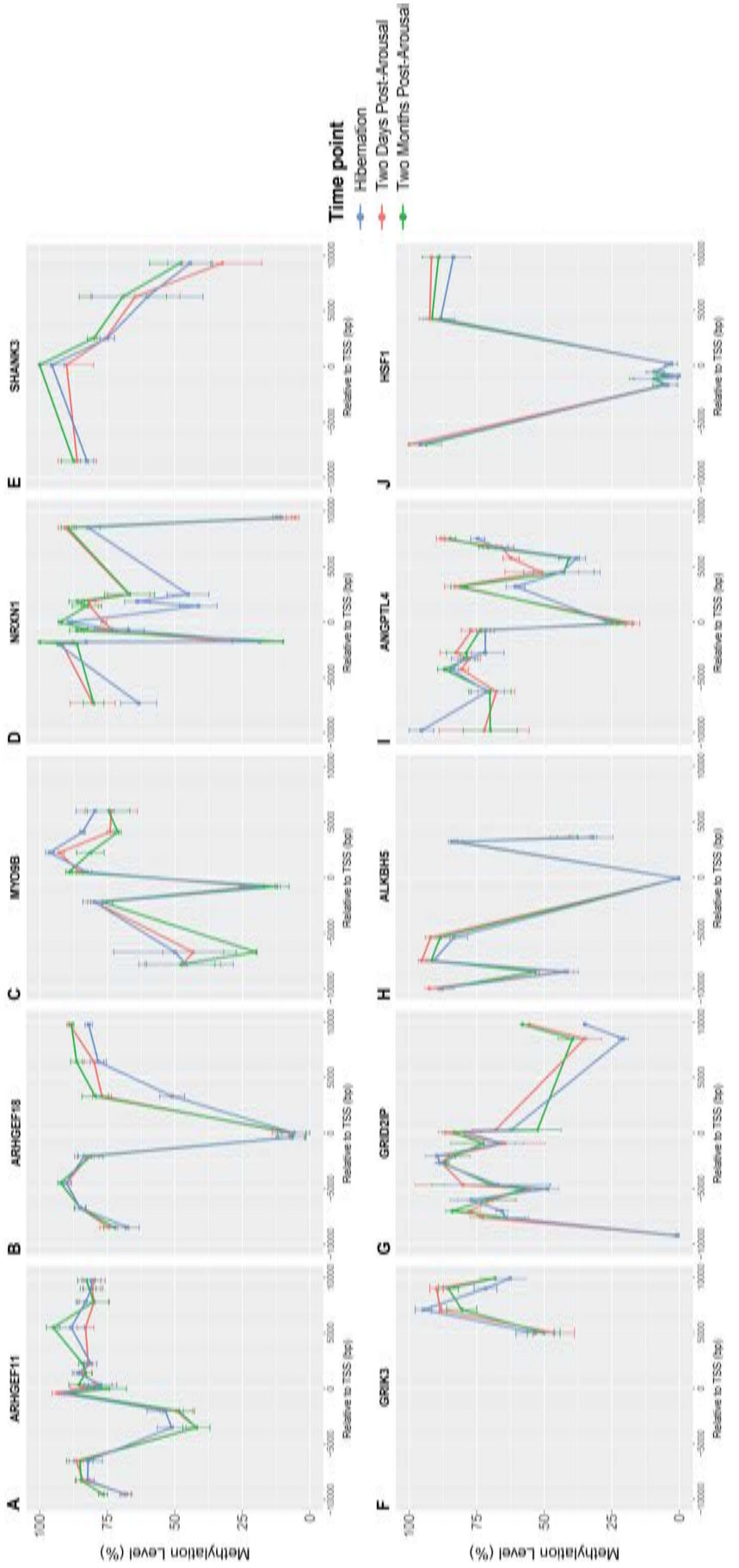
Time point
Hibernation
Two Days Post-Arousal
Two Months Post-Arousal
A
ARHGEF11
B
ARHGEF18
C
MYO9B
D
MRDN1
E
SHANK3
F
GRIK3
G
GRID2IP
H
ALKBH5
I
ANGPTL4
J
HSF1
Methylation Level (%)
Relative to TSS (bp)

Figure 4-4. Mean CpG methylation across the transcription start site of genes of interest in brain. Mean CpG methylation at regions of high CpG density across ±10 kb of the transcription start site. Error bars represent standard error of the mean of each clustered CpG region.

The RhoA signalling pathway genes *ARHGEF11*, *ARHGEF18*, and *MYO9B* were analysed. In brain, there were regions of distinctly different CpG methylation levels (**Figure 4-4, panels A-C**). For *ARHGEF11*, -9 kb from the TSS CpG methylation was 8% lower during hibernation compared to two months post-arousal, but not two days post-arousal (**Figure 4-4A**). CpG methylation of *ARHGEF18* was consistent in the promoter region brain for the three time points, but was significantly lower during hibernation than at both post-arousal time points at +3.3 kb (~27%) and +9.8 kb (8% lower) from the TSS (**Figure 4-4B**). CpG methylation of *MYO9B* was consistent at the TSS between in brain at all time points. However, during hibernation methylation was higher at -6.7 kb (31% higher) and +4.2 kb from the TSS (13% higher) compared to two months post-arousal (**Figure 4-4C**).

CpG methylation across the TSS of genes involved in NMDAR and glutamate signalling (*NRXN1*, *GRIK3*, *SHANK3*, and *GRID2IP*) was explored for all brain time points (**Figure 4-4, panels D-G**). *NRXN1* CpG methylation was lower during hibernation than two days post-arousal from the TSS to +8 kb (**Figure 4-4D**). For *GRIK3* there was an increase in CpG methylation during hibernation at +7 kb from the TSS, and reduced methylation +9.5 kb from the TSS compared to the two post-arousal time points (**Figure 4-4E**). For the hypoxia response genes (*ALKBH5, ANGPTL4*, and *HSF1*; **Figure 4-4, panels H-J**), the most noticeable difference in methylation across the TSS was in *ANGPTL4* (**Figure 4-4I**). At -9.5kb from the TSS CpG methylation was 24% higher during hibernation

compared to both post-arousal time points, and 22% lower at +3 kb from the TSS CpG methylation during hibernation compared to both post-arousal time points.

In skeletal muscle, the methylation profiles across the TSS was assessed for *ACTN3*, *BCR*, *CGNL1*, and *TNXB* (**Figure 4-5, panels A-D**). *ACTN3* was hypermethylated by 22% at -4.5 kb from the TSS during hibernation compared to two months post-arousal (**Figure 4-5A).** Hypomethylation (15%) was observed at the TSS of *TNXB* during hibernation compared to two days post-arousal (**Figure 4-5D**).

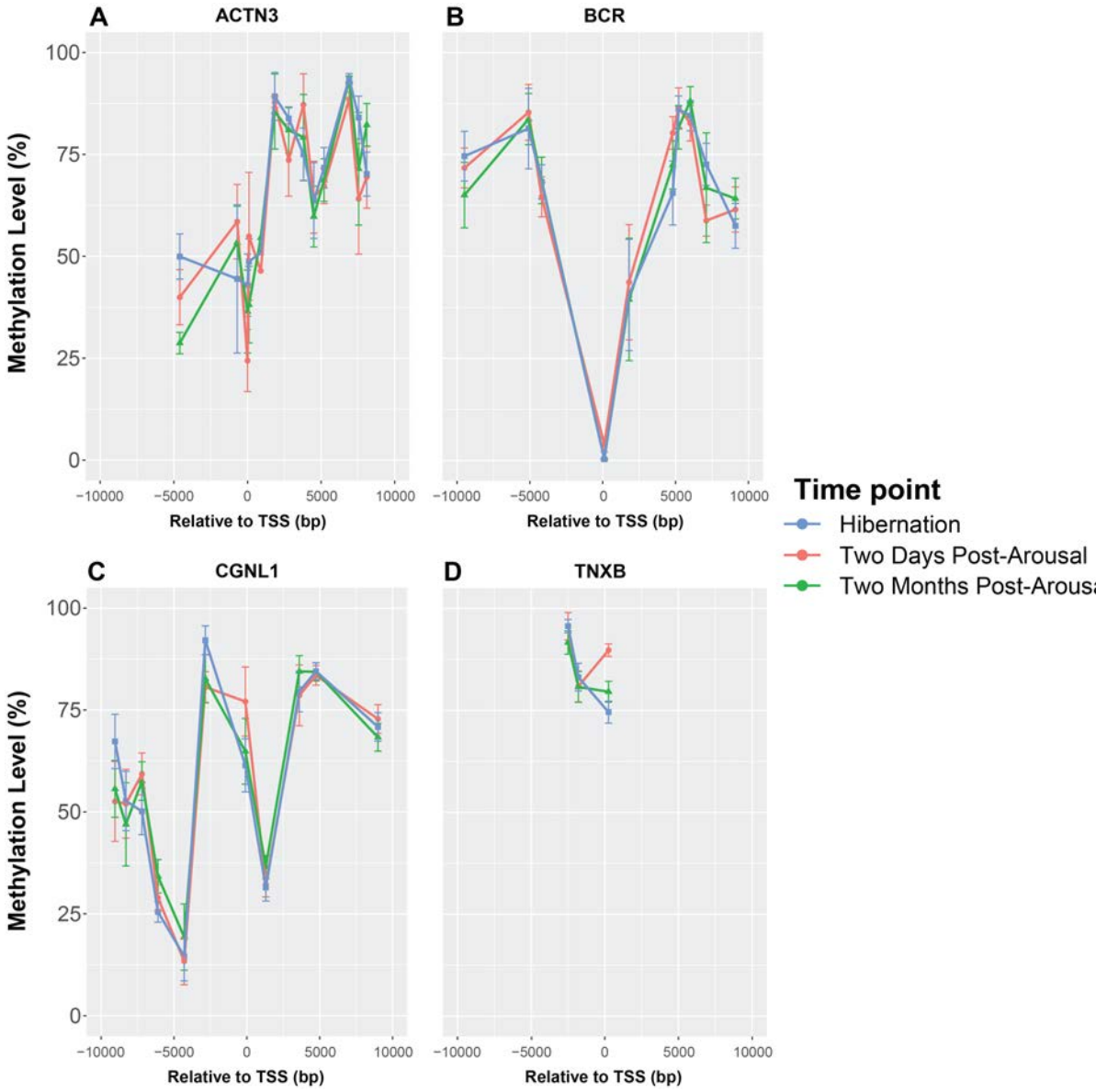

Figure 4-5. Mean CpG methylation across the transcription start site of genes of interest in skeletal muscle. Mean CpG methylation at regions of high CpG density across ±10 kb of the transcription start site. Error bars represent standard error of the mean of each clustered CpG region.

4.2.4 Discussion

The necessity for epigenetic control of adaptive processes such as hibernation is becoming increasingly evident. The role of DNA methylation in regulating hibernation has been previously explored; however, high-throughput techniques such as bisulfite sequencing have not been employed. In this study, for the first time we provide a single nucleotide resolution map of the methylome of an animal at different time points of the hibernation season. We found that CpG methylation remains unchanged globally. Rather, there is fine scale change at specific gene promoters and gene bodies. Furthermore, changes in methylation were correlated with mRNA expression. Our results suggest that CpG methylation does indeed regulate gene expression that is important for tissue-specific responses during hibernation.

4.2.4.1 Global changes in CpG methylation during hibernation

In hibernating bearded dragons, there was no significant global or meta-genomic feature CpG methylation change compared to both post-arousal time points in either brain or skeletal muscle (**Figure 4-1**). In hibernating mammals, global DNA methylation reduced by 25% in skeletal muscle and increased by 1.7-fold in brown adipose tissue when compared to euthermia [103]. The disparity between bearded dragons and mammals may be due the inability for assay-based analyses to distinguish cytosine methylation context. Therefore, the large changes in mammals could reflect DNA methylation that was detected in both the CpG and non-CpG contexts. Furthermore, RRBS does not distinguish 5hmC from 5mC, whereas assay-based methods do. 5hmC is relatively abundant in the brain [310], but it only represents ~8 to 14% of the total 5mC abundance in brain and ~3% in skeletal muscle of mouse [311]. Therefore, while these issues may be responsible

for some discrepancy, it would unlikely account for all of the difference between the mammalian studies and data presented here.

The observations here suggest that rather than a genome-wide increase in CpG methylation during hibernation (which would presumably reduce transcriptional output), there are more nuanced gene-specific changes in CpG methylation to regulate key genes necessary for bearded dragon hibernation. These gene-specific changes were observed when comparing the two post-arousal time points. The large number of differentially methylated genes between hibernation and two days post-arousal suggest that DNA methylation changes occur quickly after arousal, however, it appears more time is required to reach the "normal" methylation profile (represented by the two months post-arousal individuals).

Interestingly, the number of differentially methylated promoters and gene bodies appears to be greater between late hibernation and two days post-arousal, than between late hibernation and two months post-arousal (**Table 4-1 and 4-2**). This is surprising as the two days post-arousal time point would presumably reflect a transition state between hibernating and fully aroused animals. It could be that emergence from hibernation requires specific methylation patterns at genes required for proper arousal. This pattern is then lost as bearded dragons return to normal post-arousal physiology.

4.2.4.2 Modulation of CpG methylation may mediate neuroprotection in hibernation brains

Two genes (*RHOA* and *EPS8*) involved in the Rho signal transduction pathway are important in mediating protection from excitotoxicity in the brain during hibernation. Gene body methylation of *RHOA* increased 20% during hibernation compared to two

days post-arousal (**S4-1 Table**). In matched tissues and time points, mRNA levels were 80% higher (log_2FC 0.84; **S4-3 Table**). RhoA is part of the Rho family of GTPases and has important roles in apoptosis and excitotoxicity [312], particularly in microglia. During prolonged neuronal insult, microglia activation induces and accelerates neuronal damage through overproduction of ROS and increased glutamate release [313]. This is hypothesized to occur due to the loss of RhoA function [314]. Importantly, RhoA activation prevents excitotoxic release of glutamate in microglial cells by maintaining regulation of Src kinase activity [314], which is known to protect the brain against ischemic injury [315]. The changes in *RHOA* DNA methylation and expression may reflect changes in microglia dynamics in whole brain tissue in order to reduce the potential release of glutamate and subsequent induction of excitotoxicity.

The gene body of *EPS8* was hypomethylated ~8 kb downstream of the TSS during hibernation, which correlated with downregulation during hibernation (**S4-4 Table**). *EPS8* encodes for an actin-binding protein that interacts with the NMDAR complex and is critical to spine growth and long-term potentiation [316]. Reduced expression of *EPS8* is linked to autism spectrum disorders and gene knockout in mice results in learning and memory impairment [317]. Furthermore, *EPS8* knockout results in altered function, synaptic expression and subunit composition of NMDARs, with increased GluN2B- and reduced GluN2A-containing NMDARs [318]. Interestingly, *GRIN2B*, which encodes for GluN2B, was upregulated during hibernation in bearded dragons [268]. Increased surface expression of GluN2B is observed in Huntington's disease [319, 320] and autism is associated with GluN2B [321]. Increased expression of GluN2B subunits may reduce excitotoxicity as suppression of GluN2B results in substantial elevation of intracellular calcium, and oxygen and glucose deprivation-induced excitotoxicity [322]. Therefore,

hypomethylation of the *EPS8* gene body during hibernation may reflect a mechanism to reduce EPS8 expression, which in turn influences the NMDAR subunit composition, reducing the susceptibility of the brain to excitotoxicity.

GRIK3 was hypomethylated in the gene body, with mRNA levels decreasing greater than three-fold during hibernation (**Figure 4-4F**, **S4-4 Table**). *GRIK3* encodes for the GluK3 kainic acid receptor (KAR) subunit [323]. Much like NMDARs, KARs are excitatory glutamate receptors that facilitate Ca^{2+} influx into cells [324]. However, unlike NMDARs, little is known about their function, with roles in excitotoxicity speculative [325]. However, because KARs that contain the GluK3 subunit are excitatory and result in Ca^{2+} influx, downregulation of *GRIK3* in the brain during hibernation might limit the potential for additional excitatory synapse activation.

Many neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's disease appear to share similar physiology to brains of hibernating animals [72, 298-300]. It has been hypothesised that induction of processes such as modulation of microtubule dynamics, and NMDAR signalling disruption, increases neuroprotection in response to other stressors. Interestingly, homeobox-containing transcription factor genes, particularly *HOXA* genes, are differentially methylated in patients with AD [326]. The HOXA gene cluster on chromosome 6 of the bearded dragon is differentially methylated between the three time points in brain, with hypermethylation occurring during hibernation (**Figure 4-2**). The role of *HOX* genes in both hibernation and AD is unknown, however, the DNA methylation changes in *HOXA* genes provides further evidence that human pathological conditions and hibernation may share key response mechanisms.

4.2.4.3 Response to hypoxia in brain

Due to the reduced breathing and heart rate experienced by animals during hibernation, many organs experience ischemia (reduced blood flow) and become hypoxic [39]. The brain is a particularly sensitive organ to ischemia and hypoxia [327]. This necessitates the need for tissue-specific responses to prevent neuronal cell death. In the brains of hibernating bearded dragons, differential methylation of three key hypoxic response genes (*ANGPTL4*, *HSF1* and *ALKBH5*) may regulate this process.

ANGPTL4 is a regulator of endothelial barrier integrity and is induced by hypoxic insult [328], preventing neuronal loss and reducing swelling in ischemia stroke model mice [329, 330]. ANGPTL4 induction improves endothelial networking after stroke; preserving vasculature in the brain (reviewed in [331]). Two regions of the *ANGPTL4* gene body had decreased CpG methylation (~25%) during bearded dragon hibernation compared to two days post-arousal **(S4-2 Table**). mRNA expression of *ANGPTL4* was three-fold higher during hibernation than two days post-arousal **(S4-4 Table**).

HSF1 encodes for a transcription factor that regulates heat shock proteins (HSPs) and the heat shock response (HSR) [332, 333]. HSR is induced by several environmental stressors, including high temperatures, hypoxia and oxidative stress, resulting in expression of protein chaperones that protect misfolding and degradation of proteins [333, 334]. The gene body of *HSF1* was hypermethylated ~9.5 kb downstream of the TSS and mRNA was upregulated (**Figure 4-4J**, **S4-4 Table**). The role of HSPs in the hibernating brain has not been investigated. Increased expression of *HSF1* in the brain during hibernation may facilitate protection of proteins vulnerable to reactive oxygen species upon arousal.

ALKBH5 encodes an RNA demethylase, specifically N^6-methyladenosine (m^6A), that is induced during hypoxia by HIF-1α [335]. The consequence of m^6A is increased mRNA degradation rates [336] and stabilisation of key mRNAs during hypoxia [337]. Two hypomethylated regions exist in the promoter and gene body of *ALKBH5* and is 3.7-fold upregulated during hibernation in the brain (**S4-4 Table**). ALKBH5 aids in survival of hypoxia of various cancers by demethylating and stabilising *NANOG* and *FOXM1* mRNA [338, 339], with *NANOG* highly upregulated (8.7-fold) in hibernating bearded dragon brain [268]. The role of m^6A in hibernation has not been investigated to date, however, its function in many physiological processes suggests it could have an important role. These observations highlight that RNA methylation dynamics may be important for regulating stress responses, particularly in the brain, during hibernation.

4.2.4.4 Differential methylation may facilitate switch to slow-twitch muscle fibres during hibernation

In skeletal muscle, *ACTN3* (**Figure 4-5A**) and *MYOZ2* were differentially methylated in the promoter and gene bodies during hibernation, respectively, and mRNA of both are downregulated (**S4-1 Table**, **S4-3 Table**) [268]. *ACTN3* encodes alpha-actinin-3 and is predominantly expressed in fast-twitch type II muscle fibres, with polymorphisms associated with increased power capacity of the individual in humans [340]. *MYOZ2* encodes a protein that binds calcineurin to alpha-actinin at the sarcomere in slow type I muscle fibres, and is only expressed in slow-twitch muscle fibres [341]. However, *MYOZ2* knockout mice display increased calcineurin signalling. Increased calcineurin signalling is a key function of activated PGC-1α (which was previously shown to be upregulated in hibernating bearded dragon skeletal muscle [268]), which is important for fast-to-slow muscle fibre transformation [342, 343]. Differential methylation of these

genes may facilitate their downregulated expression and be necessary for the switch in muscle fibre type.

4.2.5 Conclusion

In this study, we present the first high-throughput investigation of DNA methylation at different time points of the hibernating season. During central bearded dragon hibernation there was no large scale global or meta-genomic CpG methylation change. Rather there were subtle changes at promoters and gene bodies of key genes. In brain, differential DNA methylation appears to regulate vital neuroprotective mechanisms including hypoxia response, prevention of excitotoxicity and apoptosis progression. Furthermore, dynamic changes in CpG methylation may regulate the switch from fast-twitch muscle fibres to slow-twitch muscle fibres in skeletal muscle; a process known to preserve muscle from atrophy. This study presents further evidence of multiple levels of gene regulation that are necessary for controlling stress response and preservation pathways involved in this remarkable adaptive phenotype.

4.2.6 Methods

4.2.6.1 Animals and tissue collection

Central bearded dragons (*Pogona vitticeps*) were captive bred and housed at the University of Canberra under a protocol approved by the University of Canberra Animal Ethics Committee (CEAE17-08) and ACT Government License to Keep (K9640). Husbandry practices fulfil the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) sections 3.2.13 - 3.2.23.

Captive conditions are as described in Capraro *et. al* (2019) [268]. All individuals are matched with that in Capraro *et. al* (2019). Commercial sources of vegetables, mice and live insects (crickets and cockroaches) were provided as food, with water available *ad libitum*. Cages were cleaned thoroughly monthly, with superficial cleaning done daily (removal of faecal matter and unused food, maintenance of clean water containers). Logs and small branches were provided as basking perches and cardboard boxes provided as retreats. Enclosures were lit by a fluorescent lamp, a strong UVB light source, and a floodlamp (as a heat source) on a variable light:dark (L:D) cycle: August – mid-June (13hL:11hD; 22°C), late June (2 weeks- 6hL : 18hD; 18°C) and winter hibernation (0hL:24hD; 12°C). For two weeks prior to hibernation, heat and light were reduced and animals were not fed. All heat and UV lights were turned off for 8 weeks and the facility temperature maintained at 12°C, which stimulated any animals remaining active to hibernate. The conditions of artificial hibernation are chosen to mimic those that occur during natural hibernation, in that ambient temperatures are dropped, and light availability reduced. Body temperatures of hibernating animals was the same as ambient temperature (12°C) due to the lack of access to heat sources. After arousal from hibernation, animals were subject to full summer conditions (13hL:11hD; 22°C). Body

temperatures of animals was at least 22°C (ambient) with the addition of access to a heat source. Whole brain and femoral skeletal muscle tissue were collected from three individuals at three time points: late hibernation, and two days post-arousal and two months post-arousal. All lizards were male. Tissues were collected immediately after euthanizing (lethal injection of sodium pentobarbitone 65mg/kg by caudal venepuncture), snap frozen in liquid nitrogen and stored at -80°C until genomic DNA extraction. All post-arousal animals were sacrificed between zeitgeber time (ZT) 3 and ZT5, where ZT0 is lights on and ZT13 is lights off. Hibernating animals were sacrificed between circadian time (CT) 3 and CT5, where CT0 is the same time of day as ZT0, however, without lights turning on.

4.2.6.2 DNA isolation and reduced representation bisulfite sequencing

Genomic DNA was extracted from 30 mg of each tissue. Tissue extracts were homogenized using T10 Basic ULTRA-TURRAX® Homogenizer (IKA, Staufen im Breisgau, Germany), and DNA purified using the DNeasy Blood and Tissue Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNAse A (20 mg/ml; QIAGEN, Hilden, Germany) was used to digest RNA. For each sample, 3 µg of high integrity genomic DNA (DIN > 9) was used to construct sequencing libraries with the NuGEN Ovation® RRBS Methyl-Seq (NuGEN Technologies, Inc., Redwood City, USA) according to the manufacturer's instructions.

4.2.6.3 Bioinformatics analysis

Raw read quality was analyzed with FastQC (v0.11.5) [256]. Trimmomatic (v0.36) [257] was used to trim the reads due to low read quality with the following options: CROP:70 SLIDINGWINDOW:4:15 MINLEN:36. Trimmed reads were mapped to the annotated

reference central bearded dragon genome [166] with Bismark (v0.18.1) [344] and Bowtie [345] with the following options: -D 20 -R 3 -N 0 -L 20. Results were fed into the coverage2cytosine function within Bismark to generate a report that counts all cytosines (both top and bottom strands) in the genome, irrespective of being covered in the experiment. Cytosine report files were input into the R package MethylKit (v1.2.4) [346] which was used for all further analyses. CpG sites with less than five counts were filtered for differential methylation analysis, and samples were normalized based on library size. Differentially methylated promoters and gene bodies were determined using the getMethylDiff function. Promoters were defined as 2 kb upstream and 200 bp downstream of the TSS. TSS regions were defined as 100 bp upstream and downstream of the TSS. Regions with q-value < 0.05 and 20% difference in methylation were considered differentially methylated. Differentially methylated regions (DMRs) were discovered using the R package edmr [347] and were intersected with gene promoters (-2 kb and +200 bp relative to TSSs) and gene bodies with the BEDtools (v.2.27.1) [348] intersect function. For CpG methylation across TSS plots, a generalized additive model with default smoothing (cubic smoothing spline) was used with the predict function in R to fit a regression line with 95% confidence intervals.

mRNA-seq data was gathered from Capraro *et. al* (2019) [268]. Gene ontology (GO) enrichment analysis and KEGG pathway analysis was performed with ShinyGO on differentially expressed mRNAs using mRNA-seq data (last accessed 19/1/20) [349]. All graphs were plotted with R (3.4.2) [263], RStudio (1.1.383) [264], and ggplot2 (2.2.1) [265].

4.2.7 Appendices

Supplementary files are not included in this chapter due to excessive length and can be accessed via the following file sharing link: https://www.dropbox.com/sh/j11fj1wgt8414xb/AAAP4g1wcbPVf46PR4A90ZNOa?dl=0.

Supplementary file legends are provided below.

S4-1 Table. Differentially methylated promoter and gene bodies. Full list of all differentially methylated promoters and gene bodies between hibernation, two days post-arousal and two months post-arousal in brain and skeletal muscle.

S4-2 Table. Differentially methylated regions intersected with promoters and gene bodies. Full list of differentially methylated regions as outputted from edmr R package that were successfully intersected with gene promoters and gene bodies using bedtools intersect.

S4-3 Table. Differentially expressed genes with differentially methylated promoters and gene bodies. Full list of differentially expressed genes that were differentially methylated in either the promoter or gene body. Differentially expressed genes were gathered from Capraro *et al*. (2019) as outputted from EdgeR. Log_2 fold change is relative to hibernation (*i.e.* > 1 Log_2FC is higher expression during hibernation).

S4-4 Table. Differentially expressed genes with differentially methylated regions that intersect with promoters and gene bodies. Full list of differentially expressed genes that contained differentially methylated regions that intersected the promoter or gene body. Differentially expressed genes were gathered from Capraro *et al*. (2019) as outputted from EdgeR. Log_2 fold change is relative to hibernation (*i.e.* > 1 Log_2FC is higher expression during hibernation).

S4-5 Table. Gene ontology enrichment analysis data of genes that contain differentially methylated regions and are differentially expressed. Full list of enriched biological pathway gene ontologies genes that contain differentially methylated regions and are differentially expressed as outputted from ShinyGO.

5 Discussion

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5.1 Thesis overview

Hibernation is a remarkable adaptive phenotype that involves metabolic depression and induction of stress response pathways to survive long periods of physiological stress and starvation during the colder winter months. Large scale changes in cellular and organ physiology is displayed, which requires diverse regulatory mechanisms. Physiological and cellular features of hibernation are conserved throughout eukaryotes, however, molecular-based research into hibernation has focused on mammalian systems.

Reptiles such as in the central bearded dragon possess a remarkable capacity to withstand stress associated with hibernation. In this thesis I investigated hibernation in the central bearded dragon from a genomics perspective by employing high-throughput technologies. These were used to gain a system-wide insight in the molecular processes that regulate hibernation. I examined four genetic and molecular profiles of bearded dragons during hibernation and after arousal in the brain, heart and skeletal muscle. These were the transcriptome and proteome (**Chapter 2**), the microRNAome (**Chapter 3**), and the DNA methylome (**Chapter 4**). In this chapter I assess the key findings of **Chapters 2-4** in the context of biological and biomedical research to provide an overview of the potential insights that may be gained through the research of hibernating animals. Finally, I discuss future directions of this work to further unravel the intricacies involved in this remarkable adaptive phenotype.

5.2 The transcriptome and proteome of reptile hibernation

In **Chapter 2** of this thesis, I examined the transcriptomic and proteomic profiles of central bearded dragons at three time points across the hibernation season in brain, heart and skeletal muscle. Hibernation was associated with large scale changes in gene expression when compared to the post-arousal time points (**Figure 2-1A, B**). The two post-arousal time points (two days and two months) were transcriptionally very similar, and therefore, two days post-arousal was omitted from the proteomic analysis. Less proteomic changes were observed between hibernation and post-arousal (**Table S2-1**). The relatively low identification of proteins compared to mRNA made correlation of mRNA and protein expression analyses difficult. As such, gene ontology (GO) enrichment analyses and gene set enrichment analyses (GSEA) were used to identify enriched biological pathways (**Figure 2-1C-E**). These processes belonged to two major categories: 1) gene expression and protein regulatory mechanisms; and 2) cellular stress response pathways and mechanisms (**Figure 2-1C**).

Genes involved in chromatin restructuring, miRNA-mediated gene silencing, and addition of SUMO proteins and ubiquitin were upregulated during hibernation in the brain, heart and skeletal muscle (**Figure 2-1C, D and 2-2A-D**). Of interest were the genes involved in miRNA-mediated gene silencing. The genes involved in translational repression, rather than mRNA degradation, were upregulated during hibernation, hinting at a potentially quick and efficient gene expression regulatory mechanism.

There was enrichment of stress responses common across the brain, heart and skeletal muscle (**Figure 2-2E-H**), and those that were tissue-specific (**Figure 2-4**). Common to the tissues examined, hibernation was associated with induction of the p53 stress response

pathway (**Figure 2-2E**), response to hypoxia (**Figure 2-2F**) and induction of the NF-κB signalling pathway (**Figure 2-2H**). Unique to brain was enrichment of neuroprotective processes (**Figure 2-4A and B**). In heart there was evidence for restructuring of muscles and induction of cardiac hypertrophy (**Figure 2-4C and D**). Lastly, in skeletal muscle there was enrichment of processes associated with the maintenance of muscle mass and function via conservation of mitochondrial function and protection against oxidative stress (**Figure 2-4E and F**).

5.3 microRNA-mediated gene silencing

The findings presented in **Chapter 3** provided insight into a potential role of microRNAs in mediating key biological processes in hibernating bearded dragons. The bearded dragon genome lacks annotation of non-coding RNAs including miRNAs [166]. Therefore, I predicted conserved miRNAs in the bearded dragon genome and transcriptome and discovered miRNAs novel to bearded dragons (**Figure 3-1A**). A small subset of novel and conserved miRNAs was differentially expressed during hibernation in a tissue-specific manner (**Figure 3-1B**). Furthermore, the target mRNAs of miRNAs were predicted (**Figure 3-1C**) allowing for comparison with mRNA expression from **Chapter 2**.

The data showed that miRNAs that target key gluconeogenic and fatty acid oxidation enzymes were downregulated during hibernation (**Figure 3-4B**), providing a regulatory mechanism to modulate cellular metabolism during hibernation. Moreover, miRNAs in the brain may mediate neuroprotection during hibernation by degrading key microtubule-associated protein, NMDAR signalling and apoptosis mRNAs (**Figure 3-4B**), which would cause destabilisation of NMDARs from the cytoskeleton [277, 278] and prevention

of apoptosis progression [293, 295, 297]. Further experimentation is necessary to validate the miRNA-mRNA interactions and subsequent degradation using techniques such as RNA crosslinking immunoprecipitation sequencing (CLIP-seq) and degradome sequencing [350].

5.4 DNA methylation in hibernation

In **Chapter 4**, I assessed CpG methylation in hibernating and aroused bearded dragons. To achieve this, I produced a single-nucleotide resolution map of CpG methylation in brain and skeletal muscle across three time points across the hibernation season: during late hibernation, two days post-arousal and two months post-arousal. CpG methylation did not vary globally across the genome or at meta-genomic features such as transcription start sites and promoters (**Figure 4-1**). Rather, large changes in DNA methylation was found in the promoter and gene bodies of specific genes (**Table 4-1 and 4-2**). These changes were correlated with the gene expression profiles in **Chapter 2**.

Changes in CpG methylation of promoters and gene bodies in brain correlated with changes in gene expression that suggest improved neuroprotection during hibernation (**Figure 4-4**). Two distinct responses appear to be regulated by DNA methylation: 1) prevention of excitotoxicity by blocking excessive glutamate release [314, 315] and restructuring NMDAR composition to reduce intracellular Ca^{2+} influx [318] (**Figure 4-4**); and 2) hypoxia response by promoting preservation of blood vessels (vasculature) [331], protein chaperone induction [332, 334] and stabilisation of mRNA molecules [335, 336] (**Figure 4-4**). In skeletal muscle, CpG methylation may be involved in prevention of atrophy by regulating genes that facilitate the switch in muscle fibre type (**Figure 4-5**). Changes in CpG methylation may lead to increased calcineurin signalling; a major

mechanism in fast-to-slow fibre transformation [342], and regulation of fast-twitch fibre gene expression [340].

5.5 Reptilian hibernation in the context of endothermic mammalian hibernation

For many decades it has been debated whether reptiles in fact enter the state of extended torpor during the winter known as hibernation. Hibernation was suggested to be restricted to endotherms, such as birds and mammals, that reduce their body temperature during winter torpor [156]. Many reptiles undoubtedly enter a state of metabolic depression and torpor during winter [2, 156, 162, 351, 352]. However, as reptiles do not actively regulate body temperature (ectothermic) the term 'brumation' was coined in 1965 [353].

While in some contexts it may be necessary distinguish between endothermic and ectothermic hibernation, it led to the belief the two employ entirely different biological processes. As discussed in **Chapter 1.8**, the changes experienced by hibernators exists on a continuum even in mammals and does not represent one unique phenotype. Together with the fact that hibernation is present in all classes of amniotes (mammals, reptiles and birds), endothermic and ectothermic hibernation may represent physiological processes present in a common ancestor. A core understanding of the biological processes involved in both forms of hibernation is essential for understanding its evolution. Here I attempt to bridge the gap between reptilian hibernation and endothermic hibernation by integrating the insights presented in **Chapters 2-4**.

Regulatory mechanisms that govern changes in gene and protein expression are very similar between mammals and reptiles (see **Chapter 1.6**) as shown by the transcriptomic,

miRNAomic and methylomic profiles (**Chapters 2-4**). DNA methylation (as shown in **Chapter 4**) and the histone modifying complexes such as Sin3A-HDAC (as shown in **Figure 2-2A**) affect gene expression [5, 101, 104]. miRNAs regulate the degradation of mRNA molecules (as shown in **Chapter 3**) [5, 136], and SUMO and ubiquitination proteins regulate protein function (as shown in **Figure 2-2C and D**) [5]. However, unseen in mammalian hibernators is evidence for non-cleavage translational repression via miRNAs (presented in **Chapter 2 and 3**). This would allow for an energy-efficient mechanism to halt progression of mRNA translation that can be quickly resumed after arousal from hibernation. Further experimentation would be necessary to confirm if this mechanism is active.

Cell cycle arrest which is important for metabolic depression (see **Chapter 1.4.2**) appears to be common, however, the precise pathways vary. A major regulator of cell cycle arrest is the p53 stress response pathway which was enriched during hibernation in the bearded dragon (**Figure 2-1C and E**). High expression of downstream target genes suggests that this pathway is active in bearded dragons, which does not appear to be the case in hibernating mammals (**Figure 2-2E**) [171]. Furthermore, increased histone 1 variant H1.0 and H1.3 expression (**Figure 2-3C**), which is associated with cell cycle arrest and reduced growth rates [354, 355], may be unique to hibernating bearded dragons.

Cellular metabolism is inherently different between hibernating reptiles and hibernating mammals as mammals solely use stored lipids, whereas reptiles also utilise stored glycogen (see **Chapters 1.4.3 and 1.8**). In general, glycolysis and gluconeogenesis genes and proteins were upregulated in bearded dragon during hibernation, while, surprisingly, several lipid and ketone metabolic genes were downregulated (as shown in **Figure 2-3B and Table S2-1**). Reduced expression of miRNAs appears to modulate mitochondrial

metabolism leading to the released repression of key β-oxidation and gluconeogenesis genes (as seen in **Chapter 3**). Overall, data presented in **Chapter 2 and 3** suggests that glucose remains the main source of energy during bearded dragon hibernation, with lipids having a peripheral role; such as providing glycerol as a gluconeogenic substrate. However, metabolomic analyses is required to validate these claims.

The ability for both hibernating mammals and reptiles to withstand hypoxic conditions and oxidative stress stems from the use of similar stress pathways. Hypoxia response appears to be mediated by HIF-1α in both cases (see **Chapter 1.5.1** and **Figure 2-2F**). While HIF-1α itself was not differentially expressed in bearded dragons during hibernation as it is in mammals [39], core activators and downstream targets were actively upregulated suggesting that the pathway is active (**Figure 2-2F** and **Figure 4-4**). Induction of these genes (*ANGPTL4* in particular) is thought to condition organs to hypoxia by improving endothelial networking as seen in the skeletal muscle of hibernating ground squirrel muscle [356-358]. The prepared response to oxidative stress is mediated by induction of the NF-κB signalling pathway [229-231] (as shown in **Figure 2-2H**). This results in increased expression of downstream antioxidant genes as seen in both bearded dragon and mammalian hibernators [50, 359].

Finally, evidence presented in this thesis suggests bearded dragon employs very similar organ-specific responses to mammals during hibernation. The brain of both reptile and mammalian hibernators utilise mechanisms to improve neuroprotection by limiting the potential for excitotoxicity. This is achieved by modulating microtubule and microtubule-associated protein tau (MAPT) dynamics (**Figure 2-3A** and **2-4A**) [71, 73, 74, 233], disrupting NMDAR function (**Figure 2-4B**, **Figure 3-4B** and **Figure 4-4**) [68, 360] and

prevention of apoptosis progression (**Figure 3-4B**; see **Chapter 1.6.1**). Much like in mammals [37, 87, 89-92, 239], the heart of hibernating bearded dragons undergoes cardiac remodelling to increase stroke volume and contractile strength by expressing cardiac-specific transcription factors and modulating actin cytoskeleton dynamics (**Figure 2-3E** and **2-4D**). Significant skeletal muscle atrophy is prevented by preservation of mitochondrial function, and restructuring of muscle fibres from the less fatigue resistant fast-twitch type II fibre to the more fatigue resistant slow twitch type I fibre (**Figure 2-3J, 2-4F** and **4-5**) [94-96].

Overall, the study of the molecular dynamics involved in bearded dragon hibernation presented in this thesis has shown that while reptilian and mammalian hibernation are distinct, they employ very similar biological processes that may be mediated by different mechanisms. These processes do not appear to be novel to either class. Rather, both forms of hibernators appear to utilise conserved stress and adaptive responses.

5.6 Hibernation as a biomedical resource

5.6.1 Hibernation is organism-wide preconditioning to cellular stress

Throughout this thesis I have presented cellular stress response pathways and mechanisms that appear to be induced during hibernation in bearded dragon. The importance of these genes or mechanisms in hibernation was speculated by comparing to known functions in non-hibernators. Many of these studies describe techniques that induce or reduce expression of genes and proteins which consequently resulted in protective outcomes. An example of this is *PARP1* expression which appears to be controlled by miRNAs in hibernating bearded dragons (see **Chapter 3**). Artificial *PARP1* downregulation is known to produce anti-apoptotic affects in mice models [297]. This

reinforces the idea that hibernators have not evolved unique stress response pathways. Rather, hibernators have evolved stress response control mechanisms that allows for preparation of tissues and organs to the cellular stress that will eventually occur. This prepared response, or preconditioning, allows for stress response pathways to be more effectively induced and quickly responsive.

The idea of preconditioning tissues or organs to cellular stress to induce resistance to future insult is not new. In 1986, it was discovered that induction of brief periods of ischemia (restricted blood flow to tissues) to myocardium prior to sustained periods ischemic insult delays cell death and reduces infarction size (cell death caused by inadequate blood flow) in open-chest dogs [361]. This process is known as ischemic preconditioning (IPC) and has beneficial effects on the brain, heart, liver and kidney [362-365]. IPC provides protective effects at two windows: for 2-3 hours post-IPC; and between 24 hours and 72 hours post-IPC [366]. Interestingly, both mammalian and reptilian hibernators share responses to IPC in the brain [109, 366, 367]. Hibernating and IPC brains both induce the NF-κB signalling pathway and associated antioxidant genes, in addition to increasing SUMO protein expression (presented in **Chapter 2**).

Given the similarities between IPC and hibernation, hibernators may possess additional preconditioning responses to cellular stress. These preconditioning responses would occur either organism-wide or in a tissue- and organ-specific manner. Additionally, this protection must be sustained throughout the whole hibernation period. An example of this could be preconditioning skeletal muscle to future atrophy events, such as long periods of bed rest or long-distance space travel (see **Chapter 1.10**). Alterations of muscle-specific processes and genes presented in **Chapter 2 and 4** and those in mammalian hibernators [60, 90, 94, 99, 139, 173, 368] may hold the key to understanding pathways

that can be induced prior to insult in humans to reduce the potential for atrophy. Therefore, the continued study of hibernating animals could be vital in uncovering other preconditioning strategies that may ultimately be applied to the context of human medicine.

5.6.2 Hibernation as a model to understanding pathological conditions

In addition to preconditioning animals to future cellular insult, mechanisms induced during hibernation allow for deleterious secondary pathways to be managed properly. Several physiological responses observed in hibernation appears to mirror that of pathological conditions, including neurodegenerative conditions known as "tauopathies" and cardiac hypertrophy (see **Chapter 1.6.1** and **1.6.2**). The similarities between bearded dragon during hibernation and these pathological conditions was evident in the data presented throughout this thesis. Responses that were analogous to tauopathies was observed in **Chapter 2-4**, while responses like cardiac hypertrophy was observed in **Chapter 2**.

In tauopathies such as Alzheimer's disease, MAPT is excessively phosphorylated causing disassociation from microtubules and aggregation with other tau proteins intracellularly, forming PHF and NFTs [299] (see **Chapter 1.6.1**). Aberrant tau is then able to spread extracellularly through the brain, serving as a template for normal tau proteins to become pathological [369]. Interestingly, most patients that suffer from tauopathies possess wild-type *MAPT* [370]. Furthermore, repetitive traumatic brain injury can lead to development of chronic traumatic encephalopathy (CTE), which involves hyperphosphorylated tau and deposition of NFTs [371]. This suggests that aberrant tau may accumulate is response to underlying stress rather than specific genetic conditions.

The precise reason for tau hyperphosphorylation is unknown, however, it is speculated to provide neuroprotective effects [70, 72, 75, 76, 372]. The reduced affinity of hyperphosphorylated tau for microtubules is thought to disrupt NMDAR anchoring [71]; potentially blocking progression of excitotoxicity (see **Chapter 1.6.1**). This is aligned with what is observed in the brains of hibernating animals (as shown in **Chapter 2**) [71-74]. However, unlike in tauopathies, tau protein expression is downregulated during hibernation (**Figure 2-3A**) and hyperphosphorylation is reversed upon arousal [71-73]. Downregulation and knockout of *MAPT* decreases pathological tau levels and halts neuronal loss in mice even if administered well after tau deposition [373]. This further blocks cognitive decline normally associated with tau hyperphosphorylation [374]. Furthermore, *MAPT*-deficient mice are protected from excitotoxic damage [238]. Hibernators also induce mechanisms that provide additional protection from excitotoxicity and progression of apoptosis, such as blocking excessive glutamate release, reducing expression and restructuring NMDARs, and miRNA-mediated decay of key NMDAR and apoptosis mRNAs (as seen in **Chapter 2-4**).

For these reasons, hibernators provide excellent models to study pathological conditions that occur in humans. The similarities between hibernation and conditions such as tauopathies and cardiac hypertrophy may allow for the development of early-detection methods as induction and progression of these pathological conditions may follow similar pathways. Also, if these conditions are in fact induced as protective measures, then understanding the potential reasons for their induction in hibernators could permit understanding of the underlying causes of their pathological human counterparts.

5.7 Future directions

The molecular insights gained in **Chapters 2-4** of this thesis only represent the beginning of our understanding of the molecular dynamics of hibernation in reptiles. Many of the results presented in this thesis opens new questions into the biology of hibernation and adaptive responses in general.

Firstly, several experiments could gain statistical and biological significance with validation and downstream experiments. For **Chapter 2**, differentially expressed mRNAs and proteins could be validated through real-time quantitative polymerase chain reaction (RT-qPCR) and western blot experiments, respectively. For **Chapter 3**, differentially expressed miRNAs can also be validated with RT-qPCR. Furthermore, knock-in and knock-down experiments could be used to validate whether differentially expressed miRNAs truly regulate the expression of their target mRNAs in the context of central bearded dragon hibernation.

Analysis of the transcriptome and proteome of hibernating bearded dragons as presented in **Chapter 2** shows large scale changes in gene and protein expression in brain, heart and skeletal muscle. However, these changes were only determined for the whole gene, i.e. all RNA fragments that align to any exonic region of a gene were considered equal. Therefore, variations in mRNA structure or alternative splice variants were not detected. Alternative splicing is the process whereby specific exons are included or excluded from the final mature mRNA product to increase diversity of protein products [375]. mRNA molecules that are transcribed from the same gene could potentially encode for proteins with profound differences in function. An example of this is the caspase-2 gene which encodes for both a pro-apoptotic and an anti-apoptotic isoform by alternative splicing

[376]. Furthermore, investigation of internal ribosome entry site (IRES) elements in key genes during hibernation may allow for further discovery of alternative forms of translation. The role of alternative splicing has not been directly investigated in any hibernating animal to date. Given the role of alternative splicing in increasing protein diversity throughout eukaryotes in general [377] and in stress and adaptive responses of animals [378-381], it is likely that hibernators also take advantage of this regulatory process. Future research into this topic would undoubtedly expand our understanding of the phenotypic responses involved in hibernation.

A growing field of biological research is 'epitranscriptomics'; the study of RNA modifications [382]. These modifications include various forms of methylation, such as N^6-methyladenosine (m^6A), N1-methyladenosine (m^1A) and 5-methylcytosine (m^5C), as well as adenosine-to-inosine (A-to-I) modifications. In **Chapter 4** I presented differential methylation and expression of *ALKBH5*, which encodes for an RNA m^6A demethylase that is induced by hypoxia [335]. m^6A is thought to modulate mRNA degradation rates [336], however, its role in hibernation has not been investigated. A-to-I modifications has been investigated in hibernating ground squirrels where 1,205 genes were edited after prolonged cold exposure [383]. Most of these edits were made in non-coding regions, particularly in SINE-derived repeats, where they form double-stranded bonds to stabilise RNA rather than diversify the proteome. This study and data presented in this thesis suggest that the epitranscriptome may have vital roles in regulating the responses observed in hibernators and that future studies are necessary.

In **Chapter 4** I presented the dynamic changes in the central bearded dragon methylome during hibernation. Here I only assessed the 5mC DNA modifications between hibernators and post-arousal animals. As described in **Chapter 1.7.1**, CpG methylation

can exist in several intermediate states. Future investigations of these intermediates, particularly 5hmC, is important for understanding the role demethylation plays in hibernation. Use of sequencing technologies described in **Chapter 1.9.3** would allow for these intermediate modifications to be independently sequenced and compared.

A core function of DNA methylation is to bind MBD proteins, which modify the histone structure by recruiting histone modifying proteins [115]. To elucidate the full effect of dynamic DNA methylation during hibernation, it is important to consider the changes in histone modifications and the chromatin state of DNA in general. High-throughput sequencing technologies such as ChIP-seq can be employed to understand the genome-wide changes in histone modifications. ChIP-seq of several core active modifications, such as H3K4me3 and H3K36me3, and inactive modifications, such as H3K27me3 and H3K9me3, would provide a comprehensive overview of the role chromatin modification plays in the hibernation season. This would allow for validation of gene expression changes in chromatin remodelling complexes in bearded dragon hibernation presented in **Chapter 2**.

Of interest would be investigating the effects of the SWI/SNF chromatin remodelling complex, which is involved in producing poised (also known as bivalent) chromatin states [384]. Poised chromatin is the simultaneous presence of both active and repressive histone modifications and is essential in cell differentiation and development where it is thought to provide opposing effects on gene transcription [385]. For example, the combined presence of H3K27me3 and H3K4me3 may repress short-term expression via the RNA polymerase blocking role of H3K27me3, while also preventing long-term gene repression via the DNA methyltransferase blocking role of H3K4me3 [385]. This provides plasticity of gene expression, whereby removal of one of the many modifications

can cause varying affects. As such, investigating whether hibernation results in induction of poised chromatin states through techniques such as ChIP-seq could provide invaluable insight into the phenotypic plasticity involved hibernation.

Together with ChIP-seq, the spatial organization of the chromatin within a cell can be analysed using chromosome conformation capture (3C) techniques [386]. These techniques allow for three-dimensional interactions between genomic loci, such as promoter-enhancer interactions, to be quantified. This is achieved by crosslinking interacting DNA, followed by genomic DNA digestion, ligation of crosslinked DNA, and reversal of crosslinking. Regions of DNA that interact will now exist as short ligated sequences that can then be sequenced with high-throughput sequencing technologies, known as Hi-C [387]. This could be further combined with investigation of genome-wide chromatin accessibility using ATAC-seq [388]. The combination of ChIP-seq, Hi-C and ATAC-seq would allow for a comprehensive overview of chromatin structure dynamics across the hibernation season and how entrance, maintenance and emergence from hibernation affects it.

In **Chapter 2** I showed that the argonaute-3 protein gene *AGO3* was upregulated in all tissues during hibernation. I hypothesised that due to its involvement in miRNA-mediated translational repression that the upregulation may be evidence for this process occurring. AGO3 is also thought to have a critical role in piwi-interacting RNA (piRNA) biogenesis through the “ping-pong” mechanism [389]. piRNAs are a very large and diverse class of small non-coding RNAs that range from 26 to 31 nt in length. The majority of piRNAs are antisense sequences to transposons and have been shown to transcriptionally silence them (reviewed in [389-391]). Transcriptional repression is mediated recruitment of piRNA-guided PIWI proteins, which form heterochromatin and cause *de novo* DNA

methylation. Recent studies have also found that piRNAs may have roles that extends beyond silencing transposons [391]. piRNAs have been shown to guide PIWI proteins to destabilise mRNA targets in a miRNA-like manner during meiosis and late spermiogenesis of mice, as well as cleaving mRNA targets by conventional PIWI-dependent cleavage (reviewed in [391]). As such, the growing body of evidence for diverse piRNA functions warrants investigation in unique biological contexts such as hibernation.

5.8 Concluding remarks

In this thesis I set out to understand the molecular dynamics involved in hibernation of the central bearded dragon through the lens of high-throughput molecular biology techniques. Molecular research is lacking in reptilian hibernation. The combination of high-throughput sequencing technologies and investigation in a non-model hibernator allowed for the documentation of thousands of genes that may have important and diverse roles in regulating the hibernation phenotype. This work provides vital knowledge that helps piece together these the two similar adaptive responses. Furthermore, a deeper understanding of the interplay of multiple levels of gene regulation have in producing the physiological responses critical to hibernators was gained. The similarity of many of these responses to human pathological conditions suggests that future research could propel hibernation into the realm of human biomedical research. Before this can occur, many unanswered questions remain to be addressed. Many of these findings require validation experiments, co-analysis with other experiments and comparison with additional time points across the hibernation season. The implementation of complex molecular biological research tools would undoubtedly allow for an unprecedented understanding of this unique and remarkable phenotype in the years to come. The future of hibernation research looks very promising.

6 References

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7.1 Paper I

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RESEARCH ARTICLE Open Access

Waking the sleeping dragon: gene expression profiling reveals adaptive strategies of the hibernating reptile Pogona vitticeps

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Abstract

Background: Hibernation is a physiological state exploited by many animals exposed to prolonged adverse environmental conditions associated with winter. Large changes in metabolism and cellular function occur, with many stress response pathways modulated to tolerate physiological challenges that might otherwise be lethal. Many studies have sought to elucidate the molecular mechanisms of mammalian hibernation, but detailed analyses are lacking in reptiles. Here we examine gene expression in the Australian central bearded dragon (*Pogona vitticeps*) using mRNA-seq and label-free quantitative mass spectrometry in matched brain, heart and skeletal muscle samples from animals at late hibernation, 2 days post-arousal and 2 months post-arousal.

Results: We identified differentially expressed genes in all tissues between hibernation and post-arousal time points; with 4264 differentially expressed genes in brain, 5340 differentially expressed genes in heart, and 5587 differentially expressed genes in skeletal muscle. Furthermore, we identified 2482 differentially expressed genes across all tissues. Proteomic analysis identified 743 proteins (58 differentially expressed) in brain, 535 (57 differentially expressed) in heart, and 337 (36 differentially expressed) in skeletal muscle. Tissue-specific analyses revealed enrichment of protective mechanisms in all tissues, including neuroprotective pathways in brain, cardiac hypertrophic processes in heart, and atrophy protective pathways in skeletal muscle. In all tissues stress response pathways were induced during hibernation, as well as evidence for gene expression regulation at transcription, translation and post-translation.

Conclusions: These results reveal critical stress response pathways and protective mechanisms that allow for maintenance of both tissue-specific function, and survival during hibernation in the central bearded dragon. Furthermore, we provide evidence for multiple levels of gene expression regulation during hibernation, particularly enrichment of miRNA-mediated translational repression machinery; a process that would allow for rapid and energy efficient reactivation of translation from mature mRNA molecules at arousal. This study is the first molecular investigation of its kind in a hibernating reptile, and identifies strategies not yet observed in other hibernators to cope stress associated with this remarkable state of metabolic depression.

Keywords: Hibernation, *Pogona vitticeps*, Central bearded dragon, RNA sequencing, Proteomics, miRNA, Epigenetics, Stress response

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Background

Hibernation is an extreme state of inactivity used among diverse animal lineages to cope with low or unpredictable food availability and unfavourable seasonal conditions during winter. Hibernation involves long periods of hypometabolism (torpor), often interrupted by shorter periods of euthermia (interbout arousal). These periods of euthermia allow animals to rewarm and replenish gene and protein products; processes that are virtually halted during torpor [1]. Two decades of molecular studies of hibernation have focused on mammals, such as bears and squirrels [2–8], and recently marsupials [9], with little consideration of hibernation in reptiles. Debate surrounds the use of the word 'hibernation' in reptiles, with the thought that the lack of active body temperature regulation and inconsistent use of torpor necessitates an alternative term, i.e. 'brumation' [10]. However, there is large variation in physiology even between hibernating mammals, notably in tenrec [11], implying that hibernation is not one specific physiological state. As such, herein 'hibernation' will be used to describe the state of reptilian winter dormancy. Much like in mammals, reptilian hibernation also involves radical changes in behaviour and physiology [12].

In mammals, hibernation is achieved through a complex reprogramming of biological processes that leads to a drastic reduction in basal metabolic rate, transcription and translation, oxygen consumption, heart rate, and core body temperature, and an increase in physiological stress tolerance [13]. Hibernators employ general adaptive responses across all tissue types, and exhibit a range of tissue-specific responses. For example, during hibernation neuroprotective processes are activated in the brain (reviewed in [14]), contractive strength is increased in the heart [15, 16], and atrophy is limited in skeletal muscle [7, 16].

Hibernation in mammals is governed transcriptionally via chromatin modification and DNA methylation, post-transcriptionally via microRNAs (miRNAs), and post-translationally via protein modifications such as SUMOylation (reviewed in [13]). While non-cleavage translational repression of mRNAs via miRNAs is thought to be important in mammalian hibernation, as of yet, there is no direct evidence.

Exploring mechanisms used by different species to cope with extreme conditions and stressors may yield information pertinent to human disease, such as age-associated neurodegeneration, muscle atrophy, and ischemia-reperfusion injury. Studies on non-traditional model species, which have evolved different physiological strategies to cope with extreme and variable conditions, provide this critically important perspective. The Australian central bearded dragon (*Pogona vitticeps*) is an excellent model to study reptilian hibernation because the genome is sequenced [17], and hibernation that mimics natural hibernation can be easily induced in captivity. Under natural conditions, bearded dragons hibernate by burying themselves in the soil or seeking refuge in fallen logs or tree stumps [12]. Typically, hibernation occurs between May and September, the coldest months of the year, where temperatures range from 5 °C to 18 °C. While physiological studies of bearded dragon hibernation in the wild is lacking, the nature of hibernation sites (buried) suggests the lizards have reduced breathing and heart rates during hibernation, with body temperatures reflecting that of ambient temperature (as they are ectothermic). However, unlike mammalian hibernators, the central bearded dragon is not known to have interbout arousals, with rewarming achieved through basking after arousal.

We profiled gene expression using mRNA sequencing (mRNA-seq) in three tissues (brain, heart and skeletal muscle) at three time points: 1) late hibernation; 2) 2 days post-arousal (pre-feed); and 3) 2 months post-arousal. We performed label-free proteomic quantification in the same three tissues at two time points: 1) late hibernation and 2) 2 months post-arousal. Differentially expressed genes and proteins were analyzed to determine overrepresented biological pathways during hibernating and waking periods. We discovered tissue-specific pathways that protect against the stress of reptilian hibernation and provide the evidence for multiple levels of gene expression regulation that may govern the physiological changes associated with hibernation.

Results

Differential gene and protein expression

Hierarchical clustering of the 3000 most highly expressed genes discovered with RNA-seq in brain, heart, and skeletal muscle grouped samples of the same tissue (Fig. 1a). Within the tissue-specific clusters, three biological replicates of hibernating individuals were separated from the two post-arousal time points, which clustered together as a single group. Since the two post-arousal time points clustered together and differential gene expression analysis revealed minor differences in expression between the two post-arousal time points (Additional file 1: Table S1), they were treated as a single time point for all subsequent differential gene expression analyses. The greatest number of differentially expressed genes between hibernating and aroused animals was observed in skeletal muscle, followed by heart, and then brain (Fig. 1b, Additional file 1: Table S1). A subset of 1311 genes was upregulated in all tissue types during hibernation and 1171 genes were downregulated (Fig. 1b).

Considering the small differences in gene expression between 2 days post-arousal and 2 months post-arousal

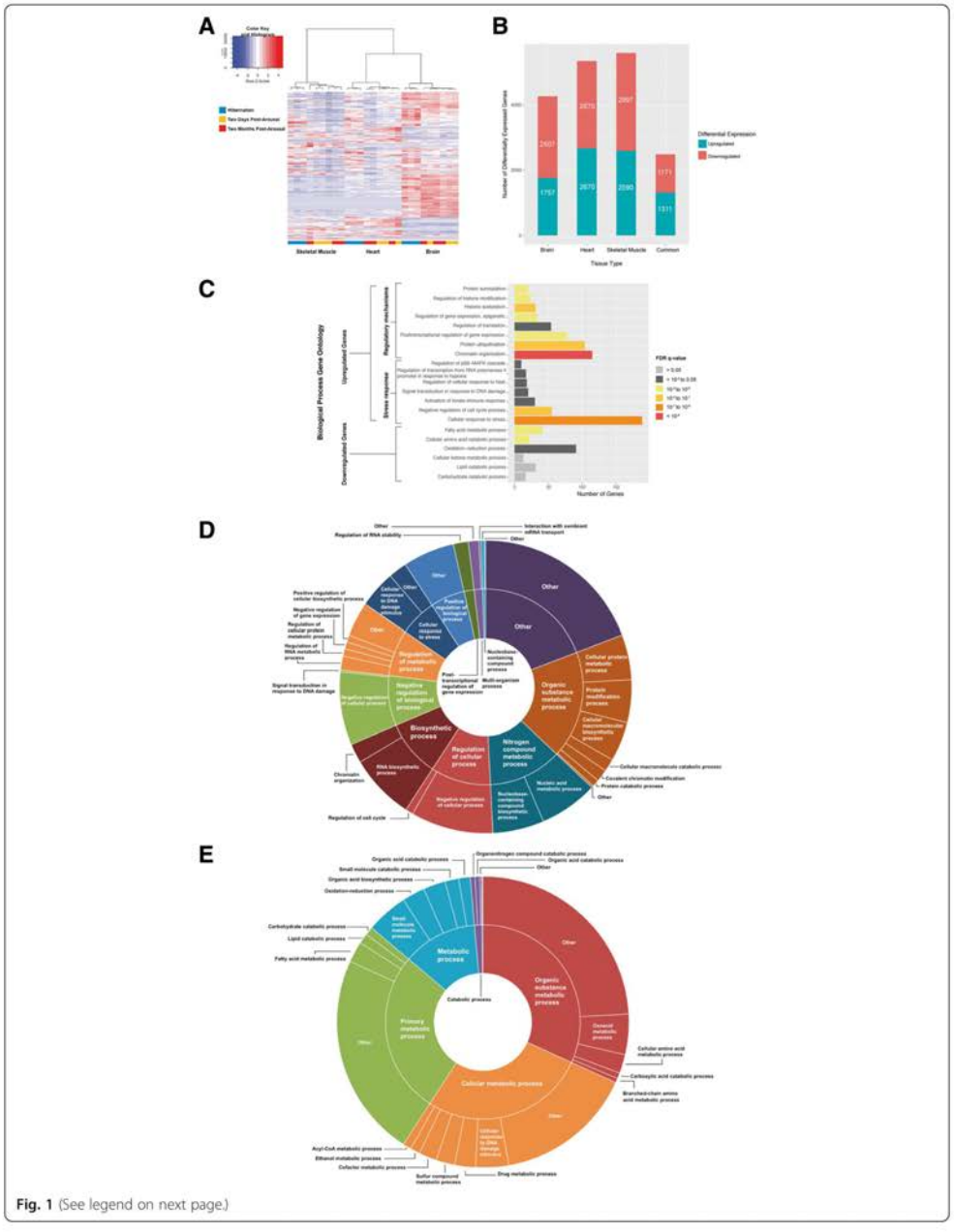

Fig. 1 (See legend on next page.)

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Fig. 1 Differential gene expression and gene ontology enrichment analysis. **a** Heatmap of the 3000 most highly expressed genes in all 27 samples with hierarchical clustering of samples. Each column represents a sample, and each row represents a gene. Each tile in the heatmap shows the normalized expression of a gene (Z-score), which was calculated by subtracting the mean expression value (counts per million) of a gene across all samples from the sample specific expression value, then divided by the standard deviation of the mean expression value of the gene. Hierarchical clustering and the dendogram were calculated using Ward's method. Colour key shows Z-score, with blue indicating lower expression and red indicating higher expression compared to the mean across all samples. **b** Bar plot of the number of differentially expressed genes during hibernation as calculated in EdgeR (Log_2 fold change > 0.585 – i.e. 1.5-fold change – and FDR < 0.05). The number of differentially expressed genes are overlaid on the bars. **c** Bar plot of selected enriched gene ontology (GO) terms of upregulated and downregulated genes common to all tissues during hibernation, with color indicating FDR (q-value) of the GO term. **d** Donut plot of significantly enriched (FDR < 0.05) biological process GO terms for upregulated genes common to all tissues in hibernating individuals. **e** Donut plot of significantly enriched biological process GO terms for downregulated genes common to all tissues in hibernating individuals. The size of each segment is relative to the number of genes that fall within the specific gene ontology in our dataset

samples (Additional file 1: Table S1), 2 days post-arousal individuals were excluded from the proteomic analysis. Across all individuals (hibernators and awake) in brain, 743 proteins were identified, with the brain-specific proteins MBP, NEFM, and ATP1A2 most abundant (Additional file 2: Table S2). Twenty-seven of these proteins were upregulated, and 31 downregulated. In heart, the most abundant proteins were the muscle-specific proteins ACT, CKM, and MYH15. Of the 535 proteins identified, 29 were upregulated, and 28 downregulated during hibernation. In skeletal muscle, 337 proteins were identified, with muscle-specific proteins (CKM, TPM2, and TNNI2) the most abundant. Twenty of these proteins were upregulated, and 16 downregulated during hibernation. Overall, the correlation between mRNA and protein expression was very limited. In brain, there were 54 differentially expressed genes in the proteome that were detected in the transcriptome. However, in the transcriptome only 14 (26%) correlated with the proteome (2 upregulated and 12 downregulated during hibernation). This low correlation was mirrored in heart. Of 50 differentially expressed genes in the proteome that were detected in the trancriptome, 15 (30%) were correlated (1 upregulated and 14 downregulated during hibernation). Finally, skeletal muscle displayed the lowest correlation between proteome and transcriptome. There were 33 differentially expressed genes in the proteome, with just two (6%) correlating with the transcriptome. This low correlation may be due to the relatively low number of identified genes in the proteome (primarily high abundance proteins) compared to the transcriptome. For example, in brain 10,000 genes were identified with a counts per million (CPM) > 10, while only 735 proteins were identified. This reflects a mere 7% of transcripts that have associated proteomic data.

Biological processes common to all tissues examined

Gene ontology (GO) enrichment analysis of the 1311 common upregulated genes in hibernators identified 259 biological process GO terms that were significantly enriched (FDR < 0.05) (Fig. 1d and Additional file 3: Table S3). These processes belonged to two major categories: 1) regulatory mechanisms of gene expression, protein translation and protein function; 2) cellular stress response and mitigation of stress severity (Fig. 1c).

Regulation of gene expression

During hibernation, gene expression appears to be regulated at transcription, post-transcription, and post-translation across all tissues examined. Chromatin organization (GO:0006325) was enriched during hibernation (Fig. 1c and Additional file 3: Table S3); with 115 upregulated genes that included members of epigenetic modifying complexes such as the Sin3a histone deacetylase (HDAC) complex, the SWI/SNF complex, the Ada2a-containing (ATAC) complex, and Polycomb-group genes (Fig. 2a). Gene set enrichment analysis (GSEA) supported this finding, with enrichment of similar biological pathways, including chromatin organization (M13550) and regulation of gene expression, epigenetic (M16267), observed in during hibernation in all tissues (Additional file 4: Table S4). While chromatin modifying proteins were not differentially expressed in the proteomic data, changes in histone protein expression were evident across all tissues. H1F0 and HIST1H1D were upregulated during hibernation in brain (Fig. 3c). In contrast, these two proteins were downregulated during hibernation in heart, whereas H4 was upregulated (Fig. 3g). In skeletal muscle, H4 and H2B1C were both downregulated during hibernation (Fig. 3i).

Regulation of translation (GO:0006417) (51 genes) was enriched during hibernation, and there were 18 genes upregulated during hibernation that modulate gene expression by miRNAs (GO:0060964) (Additional file 3: Table S3). GSEA reinforced this enrichment (gene silencing by RNA; M16422) in all tissues (Additional file 4: Table S4). Importantly, these included genes required for miRNA-mediated translational repression (Fig. 2b). Notably, the cleavage-competent RNA-induced silencing complex (RISC) subunit *AGO2* (which results in mRNA degradation) was not differentially expressed (false

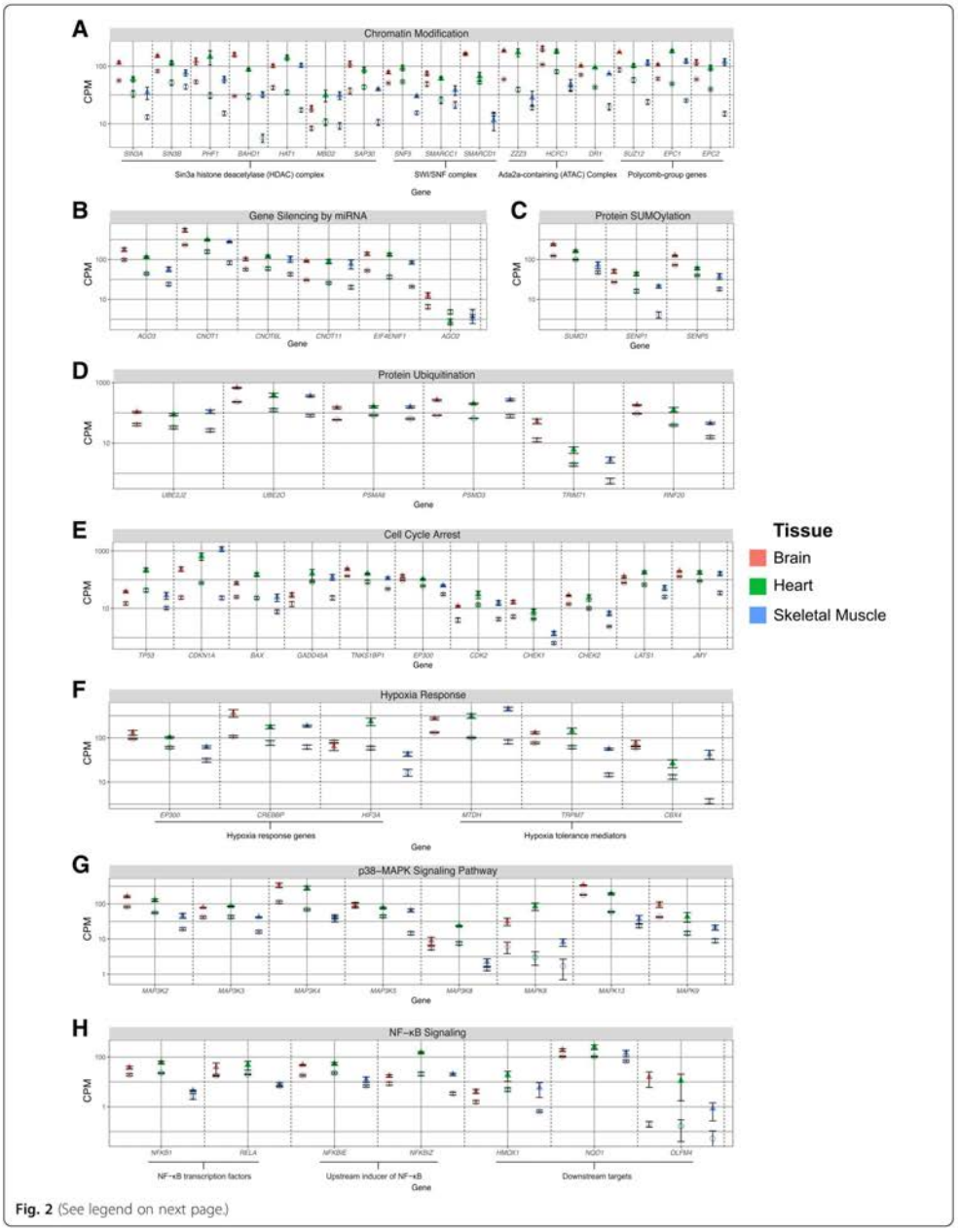

Fig. 2 (See legend on next page.)

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Fig. 2 Differential expression of genes within enriched pathways during hibernation shared by all examined tissue. Mean (across biological replicates, ± 1 standard error) expression, measured in counts per million (CPM), of genes in brain (red), heart (green), and skeletal muscle (blue). Expression is shown for hibernators (triangles) and non-hibernators (circles). Panels **a** to **d** display genes related to gene expression regulatory mechanisms. Panels **e** to **h** display genes related to stress responses. The genes are from the following biological processes: **a** chromatin modification, **b** gene silencing by miRNA, **c** protein SUMOylation, **d** protein ubiquitination, **e** cell cycle arrest, **f** hypoxia response, **g** p38-MAPK signaling pathway, and **h** NF-κB signaling. All plotted on a $\log_{10}$ scale

discovery rate (FDR) > 0.05) between hibernators and non-hibernators. Finally, during hibernation there were enrichments for both protein SUMOylation (GO:0016925) (20 genes) and ubiquitination (GO:0016567) (104 genes) (Figs. 2c and d; Additional file 3: Table S3); an observation also supported by the GSEA in all tissues (Additional file 4: Table S4).

Response to stress

Genes associated with oxidative stress, hypoxia, DNA damage and heat shock pathways were upregulated during hibernation in all examined tissues (Fig. 1c), along with 55 genes associated with negative regulation of cell cycle processes (GO:0010948) (Additional file 3: Table S3). Eleven of these 55 genes are important in the regulation of p53-mediated cell cycle arrest (Fig. 2e), including *TP53* and *CDKN1A*. Additionally, the p53-dependant G1 DNA damage response (M770) reactome pathway was highly enriched in all three tissues during hibernation (Additional file 4: Table S4). Genes that regulate transcription in response to hypoxia (GO:0061418) were also upregulated, and included the critical hypoxia response genes *EP300*, *CREBBP*, and *HIF3A*. A further three genes important for mediating

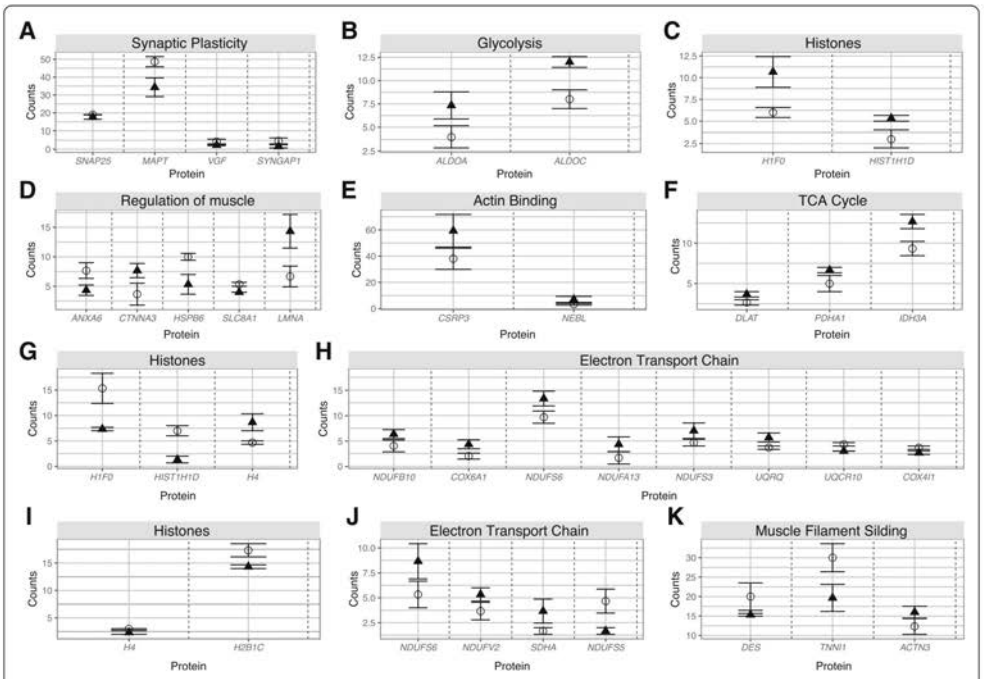

Fig. 3 Differentially expressed proteins discovered between hibernators and 2 months post-arousal individuals within tissues. Mean (across biological replicates, ± 1 standard error) normalized spectral counts of proteins. Expression is shown for hibernators (triangles) and non-hibernators (circles). Panels **a** to **c** display differentially expressed proteins in brain; panels **d** to **h** display differentially expressed proteins in heart; and panels **i** to **k** display differentially expressed proteins in skeletal muscle. See Additional file 3: Table S3 for fold-changes and full list of protein counts

hypoxia tolerance (*MTDH*, *TRPM7*, and *CBX4*) were also upregulated (Fig. 2f).

The p38 mitogen activated protein kinase (MAPK) signaling cascade is responsive to various environmental stressors [18]. Ten genes within this signaling cascade (GO:1900744), including three MAP 3Ks (MAP kinase kinase kinase), were upregulated in all tissues during hibernation. A further two MAP 3Ks and three MAPKs were also upregulated in tissues of hibernators (Fig. 2g).

Although undetected in the GO enrichment analyses, GSEA revealed that NF-κB signaling (M13738) was enriched during hibernation in all tissues (Additional file 4: Table S4). Specifically, seven genes within the NF-κB signaling pathway; a central regulator of oxidative stress response [19], were upregulated during hibernation. These included NF-κB transcription factors, upstream inducers of NF-κB signaling, and downstream target genes known to alleviate oxidative stress (Fig. 2h).

Modulation of metabolism

Modulation of metabolic genes is a common feature in hibernators [20–27]. GO analysis of the 1171 common downregulated genes revealed 44 enriched biological processes (Fig. 1e and Additional file 1: Table S1). This observation is directly supported by the GSEA, where the majority of enriched biological pathways post-arousal were related to metabolism (Additional file 4: Table S4). Enriched biological processes were predominantly related to metabolism, including: lipid catabolic processes (31 genes – GO:0016042), oxidation-reduction processes (91 genes – GO:0055114), and carbohydrate catabolic processes (16 genes – GO:0016052) (Additional file 3: Table S3). Furthermore, we observed a downregulation of three key ketone metabolic genes (*BDH2*, *ACAT1*, and *OXCT1*), which are necessary for metabolism when liver glycogen is depleted (Additional file 1: Table S1). Downregulated carbohydrate catabolism genes were predominantly related to glycoprotein and glycolipid metabolism (*NEU1*, *NEU2*, *ENOSF1*, and *NAGA*), glycosyl metabolism (*AGL* and *MAN2C1*), and galactose metabolism (*GALE* and *GALT*) (Additional file 1: Table S1).

Enriched GO terms of common upregulated genes during hibernation related to metabolism were predominantly regarded carbohydrate metabolism, including regulation of carbohydrate metabolic process (29 genes – GO:0006109) and regulation of gluconeogenesis (11 genes – GO:0006111). Specifically, this included *PFKFB3*, which stimulates glycolysis, *GSK3A*, which controls glycogen synthesis, and *FBP1*, the rate limiting enzyme of gluconeogenesis.

Corroborating our RNA-seq results, the proteomic analysis revealed differential expression of proteins involved in metabolic processes (particularly glucose metabolism) during hibernation in all tissues. In brain, two proteins upregulated during hibernation (ALDOA and ALDOC) are critical enzymes in glycolysis (Fig. 3b). In heart, six upregulated proteins (NDUFB10, COX6A1, NDUFS6, NDUFA13, NDUFS3, and UQRQ), and two downregulated proteins (UQCR10 and COX4I1) are important in the respiratory electron transport chain. Additionally, three upregulated proteins (DLAT, PDHA1, and IDH3A) are involved in the tricarboxylic acid (TCA) cycle. In skeletal muscle three proteins (NDUFV2, NDUFS6, and SDHA), important in the respiratory electron transport chain, were also upregulated during hibernation, whereas one (NDUFA5) was downregulated.

Tissue-specific responses during hibernation

GSEA revealed an enrichment for the Kyoto Encyclopedia of Genes and Genomes (KEGG) disease pathway Alzheimer's disease (H00056) in the brain post-arousal (Additional file 4: Table S4). Three genes downregulated during hibernation (*PS1*, *PS2*, and *APOE*) are linked to early onset Alzheimer's disease (Additional file 1: Table S1). Furthermore, five genes responsible for the phosphorylation of the microtubule associated protein tau (MAPT – an important protein in the central nervous system) were upregulated during hibernation (Fig. 4a). Additionally, in the bearded dragon, we observed that two N-methyl-D-aspartate receptor (NMDAR) genes were differentially expressed during hibernation: *GRIN1* was downregulated during hibernation, and *GRIN2B* was upregulated during hibernation (Fig. 4b). Proteomic analysis revealed four proteins involved in synaptic plasticity (SNAP25, MAPT, VGF, and SYNGAP1) were downregulated in brain during hibernation (Fig. 3a).

During mammalian hibernation, transcription factors responsible for cardiac muscle development, and induction of cardiac hypertrophy, are important in maintaining cardiac function [28]. In bearded dragon, four cardiac transcription factors and *PPP3CC* (a cardiac hypertrophy regulator) were upregulated in heart during hibernation (Fig. 4c). Furthermore, genes required for cardiac remodeling and associated with actin cytoskeleton modulation were upregulated during hibernation, including three myosin genes and two actin genes (Fig. 4d). Proteomic analysis revealed 5 differentially expressed proteins (ANXA6, CTNNA3, HSPB6, SLC8A1, and LMNA) involved in regulating muscle system processes (Fig. 3d), and 2 proteins (CSRP3 and NEBL) involved in actin binding (Fig. 3e).

In skeletal muscle, the transforming growth factor beta-receptor (TGF-β) signaling pathway and bone morphogenetic protein (BMP) signaling pathway act antagonistically to balance muscle atrophy and hypertrophy [29]. We observed upregulation of three positive regulators of

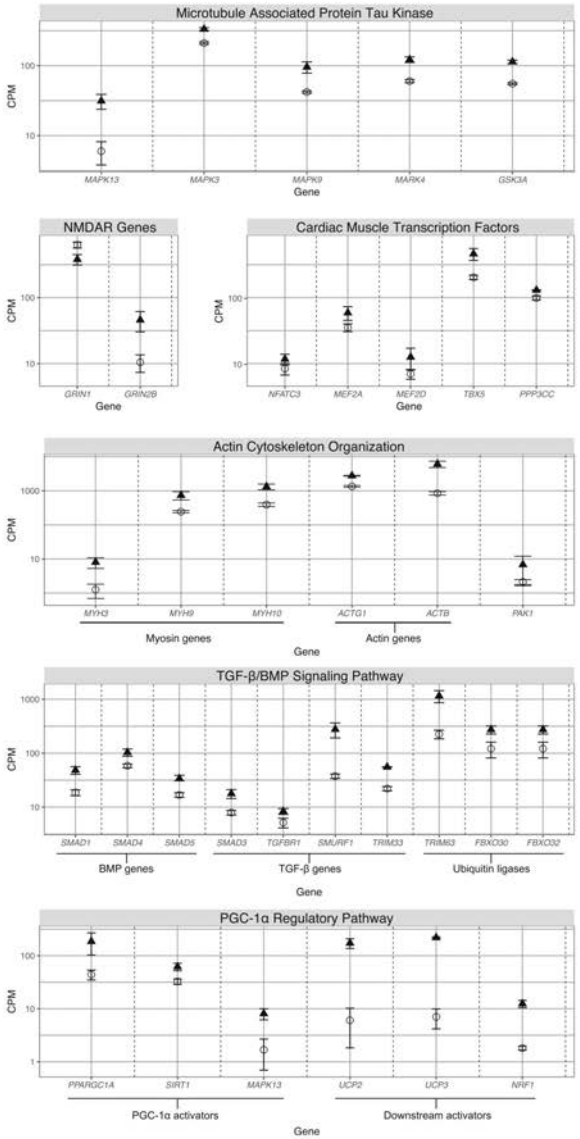

Fig. 4 Differential expression of genes within tissue-specific processes. Mean expression (across biological replicates, ± 1 standard error), measured in counts per million (CPM). Expression is shown for hibernators (triangles) and non-hibernators (circles). The genes are from the following brain-specific processes: **a** microtubule-associated protein Tau kinases, **b** N-methyl-D-aspartate receptor genes; heart-specific processes: **c** actin cytoskeleton organization, **d** cardiac muscle transcription factors; and skeletal muscle-specific processes: **e** the TGF-β signaling pathway/ BMP pathway, and **f** the PGC-1α pathway. See Additional file 1: Table S1 for fold-changes and false discovery rates

the BMP signaling pathway, and four positive regulators of the TGF-β pathway. Three muscle atrophy-related ubiquitin ligases were also upregulated during hibernation (Fig. 4e). The critical TGF-β gene myostatin (*MSTN*) was not differentially expressed between hibernating and awake animals. Finally, we observed upregulation of *PPARGCIA* (which encodes PGC-1α; the master regulator of mitochondrial biogenesis), along with two of its activators, and three downstream targets (Fig. 4f); a process which is known to be important for prevention of muscle atrophy in mammals [30]. Three differentially expressed proteins identified by proteomic analysis are involved in muscle function (DES, TNNI1, and ACTN3); particularly muscle filament sliding (Fig. 3k).

Discussion

Hibernation in reptiles is poorly studied compared to mammals. Beyond large-scale physiological responses, such as reduced heart and metabolic rate [31], the strategies common (and different) to hibernators from the two clades remain largely unknown. This study is the first to provide insight into the molecular pathways employed by a reptile during hibernation. We identified similarities between mammal and reptile hibernation, as well as responses that may be novel to the bearded dragon.

Recently, the use of steady state abundances of mRNA and proteins during hibernation has been scrutinized given that hibernation is a non-steady state condition. Some protiens do not function during hibernation as they do in steady state conditions, notably regulation of transcription by p53 [32]. However, for this study, we assume functional equivalence of biological processes during hibernation and after arousal. Additionally, transcriptomic and proteomic profiles were correlated to gain a multi-level understanding. Biological pathways (identified by gene ontology and gene set enrichment analyses) and downstream targets, rather than specific genes, were focused on to gain a more nuanced representation of physiological responses during hibernation.

Control of gene expression

Evidence for multiple levels of gene regulation in hibernating bearded dragons was observed, which is unsurprising considering that alteration of gene expression is known to be critical for hibernation in mammals [13]. Accordingly, we identified upregulation of genes involved in RISC-mediated gene silencing in all tissues of hibernating bearded dragons (Fig. 2b). Tissue-specific miRNA expression has been reported during hibernation in thirteen-lined ground squirrels [33–35], little brown bats (*Myotis lucifugus*) [36–38], monito del monte (*Dromiciops gliroides*) [6], and wood frog (*Rana sylvatica*) [39] (Fig. 5).

The CCR4-NOT complex is a master regulator of gene expression and is required for RISC-mediated translational repression via the recruitment of the translation initiation factor 4E-T [40–43]. During hibernation, we identified upregulation of three key CCR4-NOT complex subunits, in addition to *EIF4ENIF1*, which encodes for 4E-T. Furthermore, in hibernating animals *AGO2* (necessary for cleavage-competent RISC) was not upregulated, instead *AGO3*; the catalytic subunit of non-cleavage-competent RISCs [44], was upregulated (Fig. 2b). We propose that an enrichment of non-cleavage-competent RISC machinery during hibernation may represent an important gene regulatory pathway for bearded dragon hibernation.

Together with enhanced stability and polyadenylation of mRNAs [45], non-cleavage miRNA-mediated repression of mRNAs would allow for energy efficient regulation of gene expression. Upon arousal from hibernation reactivation of translation from mature mRNA molecules does not require immediate transcription and RNA processing. This would explain how bearded dragons are able to restore normal metabolic function promptly after arousal; such as if they are disturbed during hibernation, and after emergence from hibernation.

Restructuring of chromatin appears to be necessary for gene expression regulation during vertebrate hibernation. Increased expression of components of the Sin3A-HDAC complex is common to bearded dragons (Fig. 2a), hibernating squirrels and frogs [46–49] (Fig. 5). Increased expression of genes in the silencing complexes ATAC and SWI/SNF, along with Polycomb group proteins (Fig. 2a), suggests that transcriptional control during hibernation requires the complicated interaction of many epigenetic modifiers. While differentially expressed chromatin remodeling proteins were not detected in the proteomic data, differential histone expression between hibernators and non-hibernators was apparent in all tissues (Fig. 3c, g and i). The role of histone expression in phenotypic plasticity is largely unknown; however, histone expression is important in genomic stability [50, 51], suggesting a potentially important role in hibernation.

An increased expression of small ubiquitin-like modifiers (SUMO) and ubiquitination genes was also observed during hibernation (Figs. 2c and d), which are known to modulate protein function in mammalian hibernation (reviewed in [13]). Given the important role SUMOylation plays in cellular stress protection (reviewed in [52]), reversible post-translational regulation appears to be a universal mechanism involved in vertebrate hibernation and stress response.

Response to cellular stress

Cellular stress responses in the bearded dragon appear consistent with those of mammalian hibernators [53,

Molecular mechanism	Bearded dragon	Ground squirrel	Fat-tailed dwarf lemur	Bear	Bat	Monito del monte	Frog
miRNA-mediated silencing	✓	✓ [34, 35]	✗	✗	✓ [36-38]	✓ [6]	✓ [39]
Upregulation of translational repression machinery	✓	✗	✗	✗	✗	✗	✗
Sin3A upregulation	✓	✓ [46]	✗	✗	✗	✗	✓ [47]
Cell cycle control	✓	✓ [53]	✗	✗	✗	✓[54]	✗
Oxidative stress response	✓	✓ [57]	✗	✗	✗	✓ [9, 58, 59]	✗
Modulation of metabolic genes	✓	✓ [20]	✓ [21]	✓ [22]	✓ [23, 24]	✓ [9, 25]	✓[26, 27]
Reduced NMDAR function in brain	✓	✓ [66]	✗	✗	✗	✗	✗
MAPT phosphorylation in brain	✓	✓ [61]	✗	✓ [61]	✗	✗	✗
Cardiac transcription factor upregulation in heart	✓	✓ [72]	✗	✗	✗	✗	✗
PAK1 upregulation in heart	✓	✗	✗	✗	✗	✗	✗
BMP + TGF-β modulation in skeletal muscle	✓	✓ [89]	✗	✗	✗	✗	✗
PGC-1α activation in skeletal muscle	✓	✓ [93]	✗	✗	✓ [94]	✗	✗

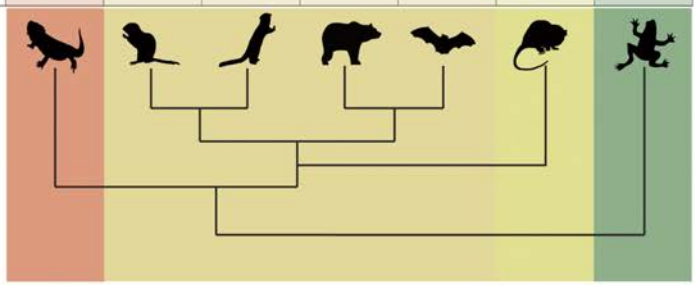

Fig. 5 Summary of molecular mechanisms utilized during hibernation by the central bearded dragon and other hibernators. For each biological process, a tick indicates there is sufficient evidence to suggest that the process occurs, whereas a cross indicates a lack of, or insufficient, evidence that the process occurs rather than not occurring. Major vertebrate clades are identified by different colors (pink – reptile, yellow – mammal, and green – amphibian) on the phylogeny. All images used are the author's own work

54](Fig. 5). Patterns of cell cycle arrest are a common observation during hibernation in thirteen-lined ground squirrels and in hypoxic red-eared slider turtles [53, 55] (Fig. 5). In bearded dragon, the p53 stress response pathway may be important in mediating this response. During hibernation in ground squirrels, Pan et al. demonstrated that while p53 localizes to the nucleus, recruits RNA polymerase II and binds DNA, the lack of target gene activation suggests that p53 does not function equivalently during hibernation [32]. However, in bearded dragons, critical target genes (including *CDKN1A*, *BAX*, and *GADD45A*) were transcriptionally upregulated during hibernation in all tissues (Fig. 2e), suggesting that p53 is indeed functional. In bearded dragons, the oxidative stress response may be guided by the NF-κB stress response pathway; much like in mammalian hibernators [56–59] (Fig. 5). Increased expression of key target genes that protect against oxidative damage (*HMOX1*, *NQO1*, and *OLFM4*) during hibernation suggests active protection from the sudden upsurge of reactive oxygen species that follows metabolic arousal from hibernation.

Our data implies that increased neuroprotection in brain during hibernation in bearded dragons may be governed by reduced N-methyl-D-aspartate receptor (NMDAR) function, ultimately preventing excitotoxicity: neuronal death by over-activation of glutamate receptors [60]. Downregulation of the NMDAR NR1 subunit gene *GRIN1* suggests lowered abundance of NMDARs at synapses, thus reducing capacity for excitotoxicity (Fig. 4b).

Increased expression of MAPT kinases in the brain could result in hyperphosphoryation of MAPT (Fig. 4a); a process that occurs in some hibernating mammals

[61](Fig. 5) and is rapidly reversed upon arousal [62–64]. Phosphorylated MAPT has reduced affinity for microtubules, which is suggested to cause disruption of NMDAR anchoring and, therefore, neuroprotection (reviewed in [65]). This process is proposed to protect against excitotoxicity in hibernating ground squirrels [66, 67], anoxia-tolerant turtles [66, 67], and hypoxia and ischemia tolerance in the brains of rats and piglets [68–70]. Notably, MAPT-deficient mice are protected from excitotoxic brain damage [71]. In bearded dragons, *MAPT* mRNA expression did not vary significantly between hibernating and post-arousal time points, however, protein expression did (Fig. 3a). The decreased abundance of MAPT protein in hibernating bearded dragons suggests that excitotoxicity prevention (via reduced receptor abundance and stability) is a critical protective measure in the brains of hibernators.

During mammal hibernation, cardiac hypertrophy increases contractile strength [15, 72]. Unlike hypertrophic cardiomyopathy disease in humans, cardiac hypertrophy in hibernators is beneficial and quickly reversed upon arousal [72]. The cardiac-specific transcription factors with increased expression during bearded dragon hibernation (Fig. 4c) have function in promoting cardiac hypertrophy [73], cardiac-specific gene expression [74], cardiac remodeling [75], and proper heart development [76]. Our data reflect those from hibernating ground squirrels (Fig. 5) [72, 77–80].

Cardiac hypertrophy requires modulation of the actin cytoskeleton and sarcomeres (the functional unit of muscle cells) [81]. This is mirrored by upregulation of actin and myosin genes during bearded dragon hibernation (Fig. 4d). Maintenance of proper cardiac function was also revealed by the higher protein abundance of CSRP3 and NEBL during hibernation (Fig. 3e). CSRP3 and NEBL bind actin and are important in maintaining muscle structure [82, 83], with mutations causing cardiomyopathy in mammals [84, 85].

Unique to bearded dragons, the important actin cytoskeletal gene *PAK1* was upregulated in heart during hibernation (Fig. 5d). PAK1 regulates excitability and contractibility of cardiomyocytes (reviewed in [86]); with over-expression improving cardiac function in mice (reviewed in [87]), and deletion worsening hypertrophic cardiomyopathy [88]. Modulating actin organization and structure appears crucial for protecting cardiac function during hibernation.

In hibernating individuals we observed upregulated pathways involved in prevention of skeletal muscle atrophy. The transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signaling pathways are antagonistic; they act to induce skeletal muscle atrophy and hypertrophy, respectively [29]. The increased expression of positive regulators of both pathways during hibernation is counterintuitive (Fig. 4e). However, both pathways modulate common targets (e.g. SMAD4 and the Akt/mTOR signaling cascade), and it has been suggested that normal maintenance of muscle mass results from precise regulation of both pathways [29]. In ground squirrels, members of both the TGF-β and BMP signaling pathways are also upregulated during hibernation (Fig. 5) [89].

PGC-1α (encoded by *PPARGC1A*) is critical in muscle remodeling and mitochondrial biogenesis [90, 91]. Genes that activate PGC-1α have increased expression during hibernation in bearded dragons, as do downstream targets (Fig. 4f). High levels of PGC-1α reduces muscle atrophy in non-hibernators (reviewed in [30]) by maintaining mitochondrial function, limiting inflammatory responses, and reducing ROS production and oxidative damage [92]. Induction of PGC-1α during hibernation may also mediate the switch from fast-twitch to slow-twitch muscle fibers [93, 94] (Fig. 5), which is important for protecting the muscle from fatigue post-arousal. Upregulation of genes within the PGC-1α pathway during hibernation suggests that this process is occurring in bearded dragon (Fig. 4f). Moreover, the increased abundance of proteins within the mitochondrial respiratory chain during hibernation (Fig. 3j) indicates preservation of mitochondrial function. We propose that increased expression of genes within the PGC-1α regulatory pathway contributes to resistance of skeletal muscle atrophy in hibernating animals.

Conclusion

Here we conducted the first transcriptional profiling and proteomic analysis of a reptile during hibernation and post-arousal from hibernation. There was evidence of neuroprotective strategies in the brain, maintenance of heart function via hypertrophy, and protection against skeletal muscle atrophy via increased antioxidant capacity and mitochondrial maintenance during hibernation. Many protective strategies we observed in hibernating bearded dragons were consistent with hibernating mammals, suggesting that there are limited solutions available to tolerate such extreme stress at the cellular level. However, bearded dragons had responses not previously detected in mammals, including the enrichment of non-cleavage competent RISC machinery during hibernation.

Methods

Animals and tissue collection

Central bearded dragons (*Pogona vitticeps*) were captive bred and housed at the University of Canberra under a protocol approved by the University of Canberra Animal Ethics Committee (CEAE17–08) and ACT Government License to Keep (K9640). Husbandry practices fulfill the

Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) sections 3.2.13–3.2.23. Commercial sources of vegetables, mice and live insects (crickets and cockroaches) were provided as food, with water available ad libitum. Cages were cleaned thoroughly monthly, with superficial cleaning done daily (removal of faecal matter and unused food, maintenance of clean water containers). Logs and small branches were provided as basking perches and cardboard boxes provided as retreats. Enclosures were lit by a fluorescent lamp, a strong UVB light source, and a floodlamp (as a heat source) on a variable light:dark (L:D) cycle: August – mid-June (13hL:11hD; 22 °C), late June (2 weeks- 6hL: 18hD; 18 °C) and winter hibernation (0hL:24hD; 12 °C). For 2 weeks prior to hibernation, heat and light were reduced and animals were not fed. All heat and UV lights were turned off for 8 weeks and the facility temperature maintained at 12 °C, which stimulated any animals remaining active to hibernate. The conditions of artificial hibernation are chosen to mimic those that occur during natural hibernation, in that ambient temperatures are dropped, and light availability reduced. Body temperatures of hibernating animals was the same as ambient temperature (12 °C) due to the lack of access to heat sources. After arousal from hibernation, animals were subject to full summer conditions (13hL:11hD; 22 °C). Body temperatures of animals was at least 22 °C (ambient) with the addition of access to a heat source.

Whole brain, whole heart and femoral skeletal muscle tissue were collected from three individuals at three time points: late hibernation, 2 days post-arousal and 2 months post-arousal. All samples were used in the transcriptomic analysis, while only late hibernation and 2 months post-arousal samples were used in the proteomic analysis. All lizards were male. Tissues were collected immediately after euthanizing (lethal injection of sodium pentobarbitone 65 mg/kg by caudal venipuncture), snap frozen in liquid nitrogen and stored at − 80 °C until RNA and protein extraction. All post-arousal animals were sacrificed between zeitgeber time (ZT) 3 and ZT5, where ZT0 is lights on and ZT13 is lights off. Hibernating animals were sacrificed between circadian time (CT) 3 and CT5, where CT0 is the same time of day as ZT0, however, without lights turning on.

RNA preparation and sequencing

Total RNA was extracted from 50 mg of each tissue. Tissue extracts were homogenized in TRIzol reagent (Thermofisher, Waltham, Massachusetts, USA) using T10 Basic ULTRA-TURRAX® Homogenizer (IKA, Staufen im Breisgau, Germany), and RNA purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. An on-column DNase digestion was performed with RNase-free DNase (QIAGEN, Hilden, Germany). For each sample, 5–10 μg of high integrity RNA (RIN > 8) was poly-A selected. Libraries were constructed with the Illumina TruSeq Total RNA Stranded RNA kit, and 76 bp single-ended reads were generated on the Illumina NextSeq 500 platform at the Ramaciotti Centre for Genomics (UNSW, Australia). All sequence data have been submitted to the NCBI short read archive under the BioProject ID PRJNA476034.

Raw read quality was analyzed with FastQC (v0.11.5) [95]. Trimmomatic (v0.36) [96] was used to trim the reads to remove low quality bases with the following options: HEADCROP:12 CROP:62 SLIDINGWINDOW:4:15. Reads were mapped to the annotated reference genome of the central bearded dragon [17] with HiSat2 (v2.0.5) [97] using default options. HTseq-count (v0.9.1) [98] was used to count reads that overlapped genomic features with the following options: -s reverse -m union. Samples were normalized using the trimmed mean of M-values (TMM) method and differentially expressed genes was calculated with EdgeR (v3.20.8) [99] in a pairwise manner using the exact test method. Resultant *P*-values were adjusted using the Benjamini-Hochberg procedure to calculate FDR. Genes with a fold-change greater than 1.5 ($\log_2$ fold-change of 0.585) and FDR less than 0.05 were considered differentially expressed. Gene ontology enrichment analysis related to differentially expressed genes were conducted with GOrilla using the human database (GO term database last updated December 9th 2017) [100]. Unranked lists of upregulated and downregulated genes in each condition and tissues were compared to a background list. The background list only included genes that were expressed (greater than 10 counts per million) within each tissue. For differentially expressed genes common to all tissues, only genes expressed in all three tissues were included in the background list. Gene set enrichment analysis (GSEA) [101] was performed for each tissue using defaults settings. Gene sets (Collection 2: Kegg, Biocarta and Reactome; Collection 5: GO Gene sets for Biological Process, Molecular Function and Cellular Component) were downloaded from MSigDB [102]. As with the differential gene expression analysis, the two post-arousal time points were collated as a single time point. All graphs were plotted with R (3.4.2) [103], RStudio (1.1.383) [104], and ggplot2 (2.2.1) [105].

Protein extraction and mass spectrometry

Total protein was extracted from 50 mg of tissue. Tissue extracts were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA), cOmplete® and EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) using T10 Basic ULTRA-TURRAX® Homogenizer (IKA, Staufen im Breisgau, Germany).

Protein concentrations were determined using a Qubit 2.0 Fluorometer (Thermofisher, Waltham, Massachusetts, USA).

Protein extracts were analyzed at the Bioanalytical Mass Spectrometry Facility at the Mark Wainwright Analytical Centre (UNSW, Australia) using label-free quantification mass spectrometry using standard protocol. Briefly, samples were digested with Trypsin (MS Grade, Thermofisher) and separated by nanoLC using an Ultimate nanoRSLC UPLC and autosampler system (Dionex, Amsterdam, Netherlands). Samples (2.5 µl) were concentrated and desalted with a micro C18 precolumn with H_2O:CH_3CN (98:2, 0.1% TFA) at 15 µl/min and a fritless nano column (75 µm × 15 cm) containing C18-AQ media (Dr Maisch, Ammerbuch-Entringen Germany). Peptides were eluted using a linear gradient of H_2O:CH_3CN (98:2, 0.1% formic acid) to H_2O:CH_3CN (64:36, 0.1% formic acid) at 200 nl/min over 60 min. 2000 V was applied to low volume titanium union and the tip positioned ~ 0.5 cm from the heated capillary (T = 275 °C) of an Orbitrap Fusion Lumos (Thermo Electron, Bremen, Germany) mass spectrometer. Positive ions were generated by electrospray and the Fusion Lumos operated in data dependent acquisition mode (DDA).

A survey scan m/z 350–1750 was acquired in the orbitrap (resolution = 120,000 at m/z 200, with an accumulation target value of 400,000 ions) and lockmass enabled (m/z 445.12003). Data-dependent tandem MS analysis was performed using a top-speed approach (cycle time of 2 s). MS2 spectra were fragmented by HCD (NCE = 30) activation mode and the ion-trap was selected as the mass analyzer. The intensity threshold for fragmentation was set to 25,000. A dynamic exclusion of 20 s was applied with a mass tolerance of 10 ppm.

Peak lists were generated using Mascot Daemon/Mascot Distiller (Matrix Science, London, England) and imported into the database search program Mascot (version 2.6.0, Matrix Science). Search parameters were: Precursor tolerance 4 ppm and product ion tolerances ±0.5 Da; Met (O) carboxyamidomethyl-Cys specified as variable modification, enzyme specificity was trypsin, with 1 missed cleavage possible. Peaks were searched against the reference genome of the central bearded dragon [17] and a non-redundant protein database from NCBI (Jan 2015).

Raw peak data were imported into Scaffold (Matrix Science, London, England) and analysed accordingly with default settings. Normalized peak lists were imported into R (3.4.2) [103] for analysis. Proteins were excluded if there was an average of less than three spectral counts across the biological replicates in both conditions. Proteins were considered differentially expressed if the standard error of the mean spectral counts of each condition (i.e. hibernation vs. post-arousal) did not overlap.

Additional files

Additional file 1: Table S1. Differential gene expression of RNA sequencing. Full list of differentially expressed genes with FDR < 0.05 for brain, heart, skeletal muscle, and common genes as outputted from EdgeR. Log_2 fold change is relative to hibernation (i.e. > 1 Log_2FC is higher expression during hibernation). (XLSX 1302 kb)

Additional file 2: Table S2. Gene ontology enrichment analysis data of differentially expressed genes. Full list of enriched biological pathway gene ontologies in upregulated and downregulated gene datasets in brain, heart, skeletal muscle, and common genes as outputted fromx GOrilla. (XLSX 140 kb)

Additional file 3: Table S3. Protein expression data. Full list of all proteins identified with label-free mass spectrometry by Scaffold. (XLSX 82 kb)

Additional file 4: Table S4. Gene Set Enrichment Analysis (GSEA) results of RNA sequencing data. GSEA results of RNA sequencing data, containing Biocarta, KEGG, Reactome, and Biological Pathway results. (XLSX 835 kb)

Abbreviations

ATAC: Ada2a-containing; BMP: Bone morphogenetic protein; CPM: Counts per million; CT: Circadian time; FDR: False discovery rate; GO: Gene Ontology; GSEA: Gene set enrichment analysis; HDAC: Histone deacetylase; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPT: Microtubule associated protein tau; miRNA: microRNA; mRNA-seq: mRNA sequencing; MSTN: Myostatin; NMDAR: N-methyl-D-aspartate receptor; RISC: RNA-induced silencing complex; SUMO: Small ubiquitin-like modifiers; TCA: Tricarboxylic acid; TGF-β: Transforming growth factor beta-receptor; ZT: Zeitgeber time

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Availability of data and materials

RNA-seq data are available in the NCBI short read archive under the BioProject ID PRJNA476034 (http://www.ncbi.nlm.nih.gov/bioproject/476034). Mass spectrometry data are available at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011171 and https://doi.org/10.6019/PXD011171 (https://www.ebi.ac.uk/pride/archive/projects/PXD011171). Computer code for processing and analyzing sequence and mass spectrometry data is available on request.

Authors' contributions

PDW, SW, DO and AG conceived and designed the study. AC and HP contributed to the design of the study. AC performed the experiments. AC performed the computational analysis of sequencing and mass spectrometry data. HP conducted Gene Set Enrichment Analysis of sequencing data. AG provided tissue samples. AC and PDW wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Experimentation using animals was approved by the University of Canberra Animal Ethics Committee (CEAE17–08) and are in accordance with ACT Government License to Keep (K9640). Husbandry practices fulfill the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) sections 3.2.13–3.2.23.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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