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Advances and applications of environmental stress adaptation research

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ABSTRACT

Evolution has produced animals that survive extreme fluctuations in environmental conditions including freezing temperatures, anoxia, desiccating conditions, and prolonged periods without food. For example, the wood frog survives whole-body freezing every winter, arresting all gross physiological functions, but recovers functions upon thawing in the spring. Likewise, many small mammals hibernate for months at a time with minimal metabolic activity, organ perfusion, and movement, yet do not suffer significant muscle atrophy upon arousal. These conditions and the biochemical adaptations employed to deal with them can be viewed as Nature's answer to problems that humans wish to answer, particularly in a biomedical context. This review focuses on recent advances in the field of animal environmental stress adaptation, starting with an emphasis on new areas of research such as epigenetics and microRNA. We then examine new and emerging technologies such as genome editing, novel sequencing applications, and single cell analysis and how these can push us closer to a deeper understanding of biochemical adaptation. Next, evaluate the potential contributions of new high-throughput technologies (e.g. next-generation sequencing, mass spectrometry proteomics) to better understanding the adaptations that support these extreme phenotypes. Concluding, we examine some of the human applications that can be gained from understanding the principles of biochemical adaptation including organ preservation and treatments for conditions such as ischemic stroke and muscle disuse atrophy.

1. Introduction

Animals have adapted to environments far outside the bounds of what humans can survive. Particularly interesting are those that have adaptations for coping with extreme, transient fluctuations from favourable conditions to the extreme, including freezing temperatures, lack of food, lack of water, and even complete lack of oxygen. For example, freeze tolerant wood frogs (*Rana sylvatica)* survive whole body freezing every winter, a multitude of mammalian species enter hibernation when food is sparse and temperatures drop, red-eared slider turtles (*Trachemys scripta elegans*) can go without breathing oxygen for months, and spadefoot toads (*Scaphiopus couchii*) estivate for most of the year in their arid habitat. Enacting the adaptations necessary for surviving these and many other stressful environmental conditions requires extensive coordinated adjustments to cells and tissues and the study of these underlies the fascinating field the comparative biochemistry and physiology that seeks to define how animals work.

The environmental stresses and the strategies of biochemical adaptation that animals utilize reflect the challenges that selected organisms must protect against, tolerate, or harness. For this reason, when humans encounter similar problems (particularly from a biomedical context) it is often possible to look to nature for examples of how it has already been answered. For example, whole-organ cryopreservation has already been solved by various amphibians and a few reptiles (Storey and Storey, 2017). Protection against ischemic stroke and ischemia-reperfusion injury to the brain are commonplace in anoxia tolerant animals and hibernating mammals (Drew et al., 2001; Nilsson and Lutz, 1992). Hibernators also resist muscle disuse atrophy even when inactive for many months at a time (Lee et al., 2008). These examples and others illustrate just how beneficial studying diverse animals and their adaptations are to applied fields of human concern.

This review focuses on animal adaptations to environmental stresses since plants (VanWallendael et al., 2019), microorganisms (Beales, 2004; Harms et al., 2016), and insects (King and MacRae, 2015; Toxopeus and Sinclair, 2018) have been well reviewed elsewhere. With some exceptions, we focus our discussion on animals that hibernate (bears and rodents), exhibit freeze tolerance (frogs), or are tolerant to anoxia (turtles) as these are well studied and have numerous applications to other research contexts. Many aspects of the physiological and metabolic basis of animal adaptation to environmental stress have been well-studied over the past six decades, and so we look here at new areas of research, specifically studies on epigenetic and microRNA (miRNA)

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adaptations. We then look at emerging technologies and their implications for environmental stress adaptation research. Furthermore, we examine the use of high-throughput sequencing and mass spectrometry methods of investigation and how they are broadening our understanding of stress tolerance by diverse animals. Finally, applications of this research to selected biomedical fields are discussed.

2. Growing areas of research

Research on animal adaptations to environmental stress has traditionally taken an eco-physiological and metabolic approach. Animals such as the freeze-tolerant wood frog, anoxia-tolerance turtle and hibernating ground squirrels are well characterized with respect to the physiology that enable them to enter reversible states of hypometabolism, the energetics of those states, and the metabolic, enzymatic and cytoprotective mechanisms that are involved. However, questions remain about how these responses are mounted. Major advances in genome sequencing of diverse species as well as the blossoming of the field of epigenetics and the discovery of miRNA action in modulating mRNA fate have now given researchers multiple tools with which to probe much more deeply into environmental stress tolerance at gene, epigenetic, transcriptional and post-transcriptional levels.

2.1. Epigenetics

Epigenetics is commonly defined as the study of heritable changes in gene expression without modification of the DNA sequence itself. The prefix 'epi-' is Greek for "on" or "upon" and lends to the idea that epigenetic mechanisms create an additional layer of regulatory information that is "on" the genomic layer. Whereas the genomic layer gives instructions on *what* to make, the epigenetic layer controls *when* to make it. There are two substrates that this layer of regulatory information is written upon and read from: DNA (primarily cytosine residues) and histone proteins. Modifications to DNA and histones affect when a gene is expressed through a combination of changing the conformation of the DNA itself, acting as binding-ligands, blocking the action of trans-regulatory elements, or self-modifying properties such as epigenetic cross-talk (Reviewed in Goldberg et al., 2007; Handel et al., 2010; Lai and Pugh, 2017).

Epigenetic mechanisms comprise a *reader-writer-eraser* paradigm providing a dynamic way of modulating gene expression. Whereas genomic information alone can be read and written (e.g. by DNA and RNA polymerases), it lacks a mechanism to write *de novo* genomic regulatory information. This dynamic is demonstrated by the fact that epigenetic regulation is essential for processing incoming signals from the environment, producing a response in the form of a gene product, and making modifications to gene control that are flexible and sensitive to environmental change (Bagot and Meaney, 2010). Diet (Keating and El-Osta, 2015; Martínez et al., 2014; Milagro et al., 2009; Smith and Ryckman, 2015), exposure to toxins (Novo et al., 2018; Watson and Goodman, 2002), exercise (Denham et al., 2014; Horsburgh et al., 2015; Ling and Rönn, 2014), social status (Borghol et al., 2012), in utero environments (Pinney and Simmons, 2012; Ruchat et al., 2013), and natural disasters (Cao-Lei et al., 2014) are among the many life-history events that can promulgate changes to the epigenome and, thereby, to the transcriptome and phenotype of an individual. Responding to adverse or extreme environmental conditions necessitates changes to the transcriptome of species that have specialized adaptations. This has led our lab and others to explore the epigenomic contributions to environmental stress endurance (Storey, 2015) (Table 1). These mechanisms offer general methods to silence or permit gene expression on a genome-wide scale and, thus, are interesting candidates for the reorganization of gene expression in response to environmental conditions (Alvarado et al., 2014).

2.1.1. DNA methylation

DNA methylation most commonly refers to the addition of a methyl group to cytosine residues at the 5th position carbon (5 mC), typically in the context of CpG dinucleotides. This is a reversible, but chemically stable covalent modification that does not affect the interpretation of the underlying genetic code (Wang et al., 2015b). Methyl groups are donated universally from S-adenosyl-methionine (SAM), resulting in S-adenosyl-homocysteine (SAH) after transfer. The enzymes that catalyze the addition of methyl groups are DNA methyltransferases (DNMTs) (Fig. 1c). These are typically grouped as maintenance methyltransferases (DNMT1) that copy existing methylation patterns onto newly synthesized DNA strands and *de novo* methyltransferases (DNMT3A and DNMT3B) that create new patterns in response to internal or external stimuli. The boundary between the two functions is beginning to blur since DNMT1 may have *de novo* methyltransferase activity (Jeltsch and Jurkowska, 2014). DNMT3L is also a member of the DNMT family, and while it was originally classified as a DNMT, it has no catalytic function; instead it enhances and regulates the activity of other DNMTs (Kareta et al., 2006).

Studies of DNA methylation have shown that its function is tied to its position around or within the transcriptional unit. This is complicated by the fact that CpGs are distributed non-randomly throughout the genome of vertebrates. Specific regions of high CpG density are known as CpG islands (CGIs) and remain largely unmethylated. Much of the early work aimed at determining the function of CpG methylation revolved around genes with CGI containing promoters. While most CGIs remain unmethylated, specific genes have methylated promoter CGIs, which have been associated with long term suppression, as is the case in X inactivation (Csankovszki et al., 2001) and gene imprinting (Razin and Cedar, 1994). More studies are now considering the effect of intragenic methylation, specifically outside of CGIs. Gene bodies have been known to be consistently methylated, even in active genes. This seemed to contradict previous research that suggested that *all* DNA methylation had a silencing function, but more studies found that while gene body methylation does not inhibit transcription, it stabilizes elongation and suppresses spurious transcription beginning within the gene body itself (Neri et al., 2017; Suzuki and Bird, 2008). This could be a protective function to block the production of transcripts that do not originate from a primary transcriptional start site (TSS). These results point to a model where DNA methylation may block the attachment or initiation of transcription, but once transcription is initiated methylation is not an obstacle to its machinery (Neri et al., 2017). This is also suggested by studies indicating that methylation around the TSS has a highly inverse relationship to expression, blocking initiation but not polymerase extension (Brenet et al., 2011) and thus methylation near the TSS may give the strongest indication of gene expression regulation by DNA methylation.

2.1.2. Histone modification

Within the nucleus, DNA is wrapped around an octamer of histone proteins to form nucleosomes, the building blocks of chromatin. Nucleosomes are the units that form high order chromosomal structures and are what make it possible to package DNA in a reproducible and dynamically reversible fashion. Chromatin can be packaged in a condensed, heterochromatic form that generally silences the underlying genes, or in a relaxed, transcriptionally permissive euchromatic form. Which conformation occurs at any given genomic locus depends both on the chromatin remodeling proteins and the histones themselves (Reviewed in Bannister and Kouzarides, 2011). As with DNA, histones are covalently modified to alter their structure and function. The C- and N- terminal tails of each histone protein protrude from their core and can be modified post-translationally with the addition of numerous chemical moieties. The resulting effect of each modification depends on its type and placement along the histone tails, the aggregate of which has been dubbed the *histone code* (Jenuwein and Allis, 2001).

Histones can be covalently modified in numerous ways including

Table 1
Summary of studies examining epigenetic mechanisms in animals adapted to environmental stresses.

Adaptation	Species	Common name	Tissue	Epigenetic marks and modifiers	Results	Reference
Hibernation	*Tamias asiaticus*	Chipmunk	Heart, kidney, liver, lung	HP27 promoter methylation	Hibernation related protein 27 expression correlated with DNA methylation levels in tissue-specific manner, DNA methylation blocks activation by transcription factor USF	Fujii et al. (2006)
			Heart, kidney, liver, lung	HP20/25/27 promoter H3K9ac, H3K14ac, H3K4me3, HAT, HDACs and HMT binding	Active transcription marks are removed from HP20, 25, and 27 in the liver which may disrupt activation by transcription factor HNF4	Tsukamoto et al. (2017, 2018)
	Ictidomys tridecemlineatus	13-Lined ground squirrel	Liver, skeletal muscle	Global DNA methylation, DNMTs, MBDs, and *mef2c* promoter methylation	Global DNA methylation increases in muscle during hibernation, sharp increase in *mef2c* promoter methylation correlated with decreased expression during entrance into torpor in muscle	Alvarado et al. (2015)
			Skeletal muscle	H3K23ac, H3S10ph, HDAC1, HDAC4	HDAC1 and 4 protein levels increase in hibernating animals, correlated with decreased H3K23ac in muscle and increased inhibitory phosphorylation of RNA Pol II	Morin and Storey (2006)
			BAT, liver, skeletal muscle, WAT	SIRT1-7	Tissue-specific expression of SIRTs across torpor-arousal cycle and increase in total cytoplasmic SIRT activity in skeletal muscle	Rouble and Storey (2015)
			Skeletal muscle	HDAC class I/II	Increased total HDAC class I/II activity in skeletal muscle of hibernating animals	Hawkins and Storey (2017)
			Skeletal muscle	H3K9ac, H3K14ac, H3K18ac, H3K23ac, H3K27ac, H3K56ac, H2AK5ac, H2BK5ac, H4K8ac, H3S10ph	Increased acetylated H3K14, 18, and 27 during arousal from hibernation suggesting transcriptional activation in skeletal muscle	Tessier et al. (2017)
			BAT, liver, skeletal muscle, WAT	H3K9ac, HATs	Tissue-specific changes in HAT protein levels across torpor-arousal cycle, increased H3K9ac in metabolically active BAT and decreased in dormant WAT	Rouble et al. (2018)
			Liver, skeletal muscle	H3K4me1, H3K9me3, H3K27me1, H3K36me2, HMTs	Increased transcriptionally permission H3K4me1 during arousal and silencing H3K9me3 during deep torpor in skeletal muscle	Watts and Storey (2019)
Freeze tolerance	*Rana sylvatica*	Wood frog	Liver, skeletal muscle	Global DNA methylation, DNMTs	> 2-fold increase in global DNA methylation in skeletal muscle while frozen, possible novel glucose dependent regulation of DNMT activity in wood frog	Zhang et al. (2019)
			Liver, skeletal muscle	H3K4me1, H3K9me3, H3K27me1, H3K36me2, HMTs	Decreased transcriptionally permissive H3K4me1, H3K27me1 during freezing while maintaining levels of H3K9me3 in skeletal muscle, decreased H3K4me1 in liver while frozen	Hawkins and Storey (2018)
Anoxia tolerance	*Trachemys scripta elegans*	Red-eared slider	Heart, liver, white muscle	Global DNA methylation, DNMTs, MDBs	Increased global DNA methylation in liver and white muscle but not heart under anoxia	Wijenayake and Storey (2016)
			Liver	H3K4me1, H3K9me3, H3K27me1	Massive increase in H3K4me1 and activity of H3K4 methylating enzymes after 20 h anoxia exposure in liver	Wijenayake et al. (2018a)
Estivation	*Apostichopus japonicus*	Sea cucumber	Body wall, intestine, respiratory tree, skeletal muscle	Global DNA methylation	Global DNA methylation increases in intestine and respiratory tree during estivation but not muscle or body wall. Total methylation levels highest in body wall compared to other tissues	Zhao et al. (2015)
			Intestine, respiratory tree, skeletal muscle	*Cyclin B* promoter methylation	Cyclin B expression decreases during estivation in intestine and muscle, correlated with decreased methylation of promoter CpGs	Zhu et al. (2016)

Abbreviations: HP – Hibernation related protein; HAT – Histone acetyltransferase; HDAC – Histone deacetylase; HMT – Histone methyltransferase; DNMT – DNA methyltransferase; MBD – methyl-binding domain protein; SIRT – Sirtuin histone deacetylase; BAT – Brown adipose tissue; WAT – White adipose tissue.

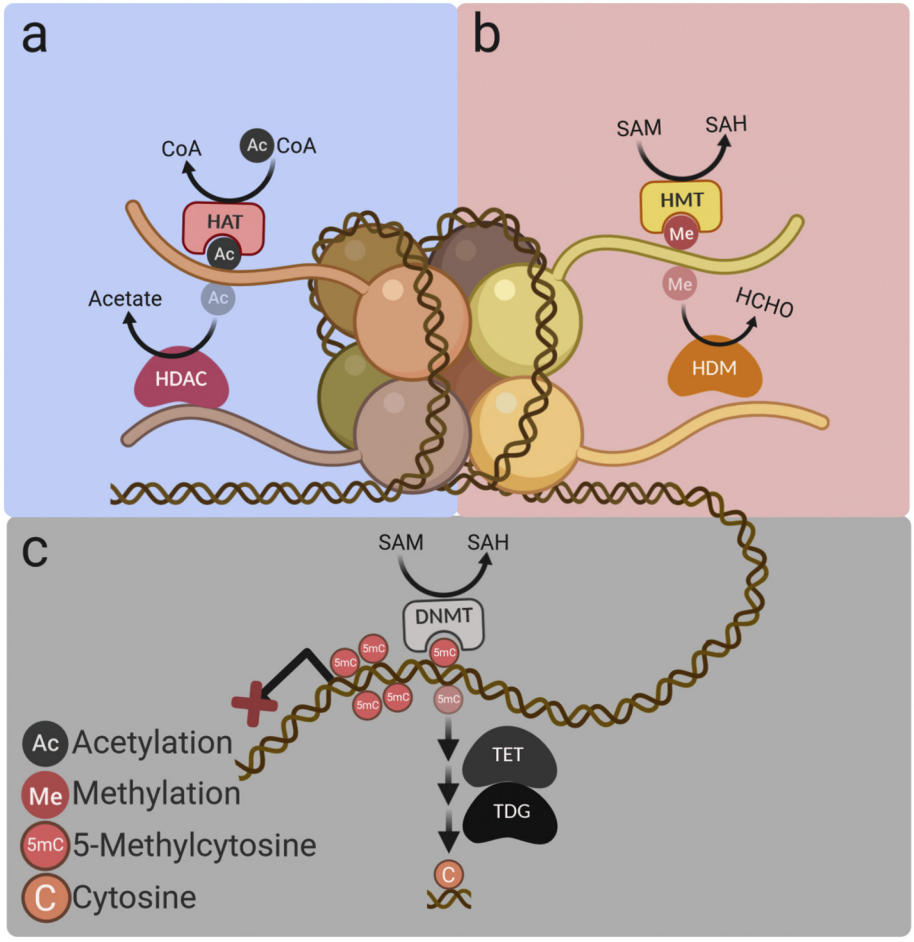

Fig. 1. Histone modifications and DNA methylation mechanisms. (a) Lysine residues on histone subunits are acetylated by histone acetyltransferases (HATs) using acetyl-CoA as an acetyl group donor. Histone acetyl marks are removed by histone deacetylases (HDACs) which results in the production of an acetate molecule. Increased histone acetylation is typically associated with transcriptional activation (b). Histones can also be methylated on lysine and arginine residues by histone methyltransferases (HMTs) using s-adenosylmethionine (SAM) as a methyl donor, resulting in the production of s-adenosylhomocysteine (SAH). The histone methyl marks can be removed by histone demethylases (HDMs) resulting in the formation of formaldehyde (HCHO). Histone methylation can result in multiple transcriptional outcomes depending on which residues are methylated. c. DNA is methylated, generally in the context of CpG dinucleotides, at the 5th carbon position of cytosines by DNA methyltransferases (DNMTs) resulting in 5-methylcytosine (5 mC) using SAM as a methyl donor. DNA demethylation occurs in multiple multi-step pathways, including serial oxidation followed by base excising repair by ten-eleven translocation (TET) and thymine DNA glycosylase (TDG) enzymes resulting in restoration of cytosine (c). DNA methylation of the promotor of a gene is generally associated with transcriptional repression.

but not limited to phosphorylation, ubiquitylation, SUMOylation, citrullination, and serotonylation but the best-studied modifications are methylation and acetylation. Lysine residues on histone tails can be acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs) (Fig. 1a). Acetyl groups are donated by acetyl-CoA and placed on the ε-amino group of lysine residues. Acetylation serves multiple functions: a) it removes a positive charge from the lysine residue, reducing the interaction between the histone protein and the negatively charged DNA backbone (Lee et al., 1993), b) it creates a binding ligand that effector proteins to can interact with (Dhalluin et al., 1999), and c) occupancy of an acetyl group excludes the addition of another modification, for example, histone H3 lysine 9 (H3K9) can be acetylated to activate transcription or methylated to silence transcription, but not both simultaneously. Together these functions generally produce a positive transcriptional outcome, and relaxation of chromatin conformation. The lysine residues of histones can also be methylated but the methyl moiety does not alter the electrostatic properties of the histone protein. This lack of intrinsic function compared to acetylation means that methylation is more dependent on effector proteins to alter transcription. Lysine methylation is catalyzed by histone methyltransferases (HMTs) (Fig. 1b), and as with DNA methylation, the sole methyl donor is SAM (Luka et al., 2009). HMTs remove the methyl group and add it to the lysine ε-amino group, making it mutually exclusive with acetylation (Seet et al., 2006). Most histone lysine methyltransferases contain a SET [Su(var)3-9, Enhancer-of-zeste and Trithorax] domain (Dillon et al., 2005), which is the main catalytic methyltransferase domain. In contrast to acetylation, up to three methyl groups can be added to each lysine residue, each with a possibly distinct function depending on the proteins that interact with it. Studies on specific methylated lysines reveal associations with transcriptional and conformation states (Kouzarides, 2002). While there are many lysines that can be methylated, the residues with strong correlations to specific transcriptional outcomes are H3K4, H3K9, and H3K27. For example, H3K4 trimethylation (H3K4me3) and H3K27me1 are present on genes that are actively expressed (Barski et al., 2007), whereas H3K9me3 is found in silenced genes often co-occurring with DNA methylation and a lack of histone acetylation (Fuks, 2005).

2.1.3. *Epigenetic contributions to hibernation and torpor*

One of the first indications that DNA methylation contributes to environmental stress adaptation in a vertebrate was in the chipmunk (*Tamias asiaticus*). Hibernation-related proteins (HPs) have been identified in this species (Kondo and Kondo, 1992), and form a 140-kDa hibernation-related protein complex that is expressed in the blood and liver of non-hibernating individuals until the animal enters hibernation at which point mRNA expression drops (Takamatsu et al., 1993). Although also present in non-hibernating species, species such as the non-hibernating tree squirrel were found not to have an active promoter for at least one member of the family, HP27, demonstrating that while the gene exists in this animal is could not be expressed (Ono et al., 2003). The tissue specific expression of this protein led one group to study the methylation pattern of the HP27 promoter in the liver and tissues where it is not expressed, namely the kidney and heart (Fujii et al., 2006). The promoter region of this gene contains CpG methylation sites that were found to be hypomethylated in the liver and hypermethylated in non-expressing tissues, consistent with the hypothesis that this gene is regulated by promoter methylation. These methylation sites were then shown to inhibit the binding of one of the transcription factors (USF) that activates HP27 expression in a methylation specific manner. Furthermore, when the authors investigated the same region of the HP27

promoter in the non-hibernating tree squirrel, they found that this animal had a base-substitution in the USF binding sequence that would have disrupted binding and thus explained the total lack of expression (Fujii et al., 2006). These results showed how DNA methylation can contribute to the hibernation phenotype by regulating this protein in a tissue specific manner. Of course, tissue specific expression of genes is only one piece of the hibernation puzzle and does not address the transcriptional reprogramming between the non-hibernating and hibernating states.

Recent studies have followed up the epigenetic regulation of hibernation-related proteins, specifically with regard to HP20, HP25, and HP27 in *T. asiaticus* (Tsukamoto et al., 2017, 2018). Similar to HP27, HP20 and HP25 are part of a 140-kDa blood complex and are specifically produced in the liver, where these two are also downregulated during hibernation. Each of these proteins have been shown individually to decrease binding of the hepatocyte nuclear factor 4 (HNF4) transcription factor to their gene promoters during hibernation. This is supported by functional assays for HP25 that demonstrated that the small heterodimer partner (SHP) inhibited HNF4 binding to the HP25 promoter in primary hepatocytes (Tsukamoto et al., 2017), HP25 being a protein that is normally upregulated during hibernation. How the change in binding of these transcriptional regulators is signalled prompted further investigation into the epigenetic mechanisms involved. Chromatin immunoprecipitation (ChIP) analysis indicated that during hibernation the active transcription marks H3K9ac, H3K14ac, and H3K4me3 each decreased on the HP proteins in question. Furthermore, analysis of the putative enzymes that would add and remove these epigenetic marks indicated that several HATs and an HMT decreased in binding to these promoters, while HDACs showed more constant expression (Tsukamoto et al., 2017, 2018). Together these results suggest that during hibernation active transcription marks on histones are removed from the HP20, HP25, and HP27 promoters which may disrupt binding of the HNF4 transcription factor, ultimately decreasing the expression of these proteins in the liver and contributing to the transition into hibernation.

Whether changes to DNA methylation patterns also occurred during the transition to hibernation itself was unknown until Alvarado et al. (2015) examined global DNA methylation levels, DNMTs, and methyl-binding domain containing proteins (MBDs) in the liver and skeletal muscle of 13-lined ground squirrels (*Ictidomys tridecemlineatus)*. Changing patterns were seen at different hibernation stages and whereas the results were an aggregate measure across all genes and could not indicate where in the genome changes to DNA methylation patterns were occurring, it was evident that changes to DNA methylation patterns are linked to hibernation. The authors chose the myocyte enhancer factor 2C (*mef2c*) transcription factor to evaluate whether DNA methylation was regulating specific genes during hibernation. This transcription factor regulates many genes involved in differentiation and was strongly downregulated in the muscle during hibernation (Alvarado et al., 2015), which is consistent with the state of dormancy where energy conservation is prioritized. Bisulfite-sequencing of this locus revealed that a series of CpG sites in the *mef2c* promoter showed a sharp increase in methylation during entry into torpor, precisely when expression falls. The authors then found that using a functional assay that methylation of the mef2c promoter was enough to silence expression and that it was not mere correlation. These results combined suggest that DNA methylation patterns dynamically respond during hibernation to regulate gene expression on a short time scale in adult tissues.

Global changes in histone modifications over the torpor-arousal cycle have also been studied in ground squirrels. Initially a comparison between non-hibernating and hibernating animals showed that the active transcriptional mark H3K23ac decreased during hibernation and some of the enzymes involved in its removal (HDAC1 and HDAC4) increased along with their activity in skeletal muscle (Morin and Storey, 2006). This is consistent with the idea that during hibernation energetically expensive transcription is globally suppressed. Although additional methods have supported the occurrence of increased HDAC activity in the muscle during hibernation (Hawkins and Storey, 2017), analysis of histone modifications and their modifiers across the torpor arousal cycle have painted a more nuanced histone-hibernation landscape.

Acetylated histone residues, which tend to support active transcriptional states, show a variety of patterns across the torpor-arousal cycle. Interestingly, whereas global acetylation levels typically decrease or remain constant in deep torpor, some acetyl-histone residues actually increase above euthermic levels during entrance into torpor and early in the arousal period (Rouble et al., 2018; Tessier et al., 2017). For example, H3K18ac increased globally by approximately three-fold during entrance into torpor, returned to euthermic levels during deep torpor, and then increased again when arousal began (Tessier et al., 2017). By contrast, H3K9ac increased only at the beginning of torpor then returned to euthermic levels for the remainder of the cycle and H3K56ac remained constant at every stage (Rouble et al., 2018). While comparatively less is known about histone modifications in brown and white adipose tissue (BAT and WAT, respectively), HAT activity and H3K9ac increased in BAT and decreased in WAT during torpor (Rouble et al., 2018), which may coincide with the physiological roles of these tissues during hibernation. BAT is responsible for non-shivering thermogenesis by oxidizing lipid stores at low levels to prevent body temperature from sinking below 0°C during torpor and at extremely high levels to power the rewarming of the body during interbout arousals. By contrast, WAT may be much less active but is still responsible for providing the main fuel, fatty acids, used over the winter months to other tissues. The intricacies of each acetyl histone residue are on display with these results and are further complicated by the enzymes that add and remove these epigenetic marks. Members of the HAT, SIRT, and HDAC families have been examined across the torpor-arousal cycle in multiple tissues (Morin and Storey, 2006; Rouble et al., 2018; Rouble and Storey, 2015), and not surprisingly a clear unified trend for levels of these proteins does not emerge, with the exception of HDAC activities that increase during torpor (Hawkins and Storey, 2017; Morin and Storey, 2006).

Histone lysine methylation was also examined in skeletal muscle and liver of ground squirrels (Watts and Storey, 2019). These tissues showed slightly different trends. Skeletal muscle showed increased permissive methyl-histone residues during entrance into and arousal from torpor whereas silencing marks increased during torpor. The liver tended to show the opposite response where H3K4me1 (a permissive modification) increased in torpor and early arousal whereas H3K9me3 (a silencing modification) decreased at all stages of torpor. These results tend to align with results from histone acetylation analyses, particularly in the muscle, as discussed above, where increased acetylation (and possibly transcription) was seen when the animal was entering or exiting torpor. While the epigenetic landscape of hibernation is not a clear as it was once thought, the results discussed here certainly suggest the importance of these mechanisms and warrant detailed investigation of these processes, particularly at the gene level.

2.1.4. Epigenetics and freeze tolerance

Hibernation is not the only environmental stress adaptation that has been studied with respect to epigenetic regulation. The freeze tolerant wood frog, *R. sylvatica*, survives full body freezing in the winter months when temperature drops below 0°C. While frozen, the blood freezes, the heart does not pump, breathing ceases, and brain activity is not detectable (Storey and Storey, 2017). Freezing protection is accomplished in part by the catabolism of liver glycogen to produce high levels of cryoprotective glucose that is distributed throughout the body at the onset of freezing. As with hibernators, wood frogs employ metabolic rate depression to conserve energy while frozen since fuel resources in each cell/tissue are finite until thawing occurs. This necessarily involves a reduction in both global transcription and translation levels (Storey and Storey, 2017), something DNA methylation could regulate.

In skeletal muscle, 5mC levels increased and 5-hydroxymethylcytosince (5hmC; the first intermediate in DNA demethylation) decreased while frogs were frozen. These changes were correlated with increased DNMT1 and DNMT3L expression (Zhang et al., 2019), the enzymes responsible for methylating DNA (Fig. 1c). Together these results paint a picture of a hypermethylation phenotype which would contribute to transcriptional silencing during freezing. The liver showed a more varied response, 5mC levels decreasing during freezing and thawing correlated with DNMT activity levels. Unlike skeletal muscle, wood frog liver maintained some activity while frozen as it is responsible for cryoprotectant synthesis even days after freezing begins (Storey and Storey, 1984), and therefore the decrease in DNA methylation in this tissue may aid this function. This study also addressed the effect of high levels of glucose on DNA methylation. High glucose is known to contribute to epigenetic dysregulation (Pinzón-Cortés et al., 2017), and therefore the wood frog, which uses extremely high glucose levels (up to 300 mM, > 50 times higher than normal) to its advantage, may show interesting glucose-DNA methylation interactions. In other studies, experimental glucose-loading of unfrozen frogs (5°C acclimated) to concentrations similar to what frogs experience during freezing led to altered DNMT enzymatic activity in the muscle (Zhang et al., 2019). These results were followed up by an in vitro assay of glucose effects on DNMT enzymatic activity in extracts of liver and skeletal muscle. Addition of 200 mM glucose to muscle extracts significantly reduced DNMT activity by about 10% in muscle and by 50% in liver extracts but did not influence the activity of a DNMT standard supplied by the manufacturer. This suggested a potentially novel wood frog specific mechanism for glucose to influence DNMT activities, although the mechanism is not yet known (Zhang et al., 2019). Further investigation is needed to determine if this glucose dependent regulation of DNMT activity has biological significance and, if it does it, whether it could be an important connection between glucose and gene expression that may help explain this frog's hyperglycemia tolerance.

As with DNA methylation, a study investigating histone methylation showed results indicating a repressive chromatin state in wood frog tissues during freezing (Hawkins and Storey, 2018). In skeletal muscle, two transcriptionally permissive histone modifications, H3K4me1 and H3K27me1, decreased to just a fraction of control values while the frog was frozen, whereas the transcriptionally repressive H3K9me3 was maintained. By removing the permissive modification and maintaining the silencing modifications, global transcription in wood frog muscle may be silenced which is consistent with a dormant state while frozen. Protein levels of multiple lysine methyltransferases were measured concurrently, and while those that target H3K4me1 either did not change across the freeze-thaw cycle or decreased, the overall enzymatic activity targeted to the H3K4 locus decreased in frozen animals, indicating potential transcriptional and post-translational regulation of this process (Hawkins and Storey, 2018). Also consistent with the DNA methylation data, histone methylation in the liver showed mixed results regarding levels of permissive and silencing methylation marks. Similar to skeletal muscle, the permissive H3K4me1 decreased whereas the silencing H3K9me3 was maintained during freezing. However, the permissive H3K27me1 was also maintained during freezing and actually increased approximately three-fold during thawing. How these results map onto regulation of individual genes is unknown, but when contrasted with completely dormant muscle, the liver maintains some metabolic function to supply cryoprotectant glucose, and a less restrictive chromatin state may be necessary.

2.1.5. Epigenetics and anoxia tolerance

While the wood frog can survive anoxia exposures separate from freezing, the vertebrate group with the highest tolerance to anoxia are various hard-shell turtles, including the red-eared slider (*T. s. elegans*) and the painted turtle (*Chrysemys picta*) that is also freeze tolerant in the northern parts of its range. Both semi-aquatic turtles spend the winter months at the bottom of ponds where oxygen levels become depleted once surface ice covers the pond and both show profound metabolic rate depression that allows them to survive for 3–4 months in cold anoxic water without breathing, all while relying on glycolysis for ATP production (with high lactate accumulation)(Jackson and Ultsch, 2010). Given the energetic cost of transcription and translation, these processes are strongly suppressed while in the hypometabolic state; e.g. protein synthesis was not measurable after 1 h of anoxia exposure in most tissues (Fraser et al., 2001). Studies of the molecular biology of turtle anoxia tolerance have focused on *T. s. elegans*, initially investigating the plausibility that global suppression of gene expression involved DNA methylation. Changes in DNA methylation were assessed after 5 h and 20 h of anoxia exposure in *T. s. elegans* liver, heart, and white skeletal muscle. Liver and white muscle showed 30% and 50% increases in global DNA methylation levels after just 5 h of anoxic submergence, whereas heart showed no change (Wijenayake and Storey, 2016). The contrast between these tissues was suggested to be due to their differing physiological functions under anoxic conditions. Whereas the liver and white muscle functions would likely be minimized in the hypometabolic state, heart is still active at basal levels (reduced to ~1 bpm under anoxia (Overgaard et al., 2007)) to circulate the blood to deliver glucose to all tissues. Consistent with the DNA methylation results, levels of MBD proteins increased in both liver and white muscle, as did DNMT enzymatic activity and protein levels of some DNMTs (Wijenayake and Storey, 2016). How these global changes manifest at the gene level has not yet been determined but the relatively large increases in global DNA methylation levels (30–50%) suggest a significant involvement of epigenetic programming to achieve gene silencing and metabolic rate depression in this animal.

Histone methylation was also investigated in *T. s. elegans* liver (Wijenayake et al., 2018a). Interestingly, both the transcriptionally permissive H3K4me1 and silencing H3K9me3 increased after 20 h of anoxia exposure, whereas the transcriptionally permissive H3K27me1 decreased. These results were matched by enzyme activities at these loci where activity at H3K4 and H3K9 both increased at the 20 h mark. These results give the first look at the histone landscape during complete anoxia exposure in a vertebrate, and while an obvious response was not seen, a more fine-tuned investigation into which genes are affected is prompted.

2.1.6. Epigenetics and estivation

While the species discussed above all enter a state of hypometabolism in response to cold-temperature dependent environmental changes, hypometabolism is also seen in animals that encounter seasonal temperature shifts above their normal range. One example is the sea cucumber, *Apostichopus japonicus*, which estivates when ocean waters increase above 25°C (Yang et al., 2006). Estivation can last as long as 4 months and, during this time, feeding and growth cease (indeed the intestine degenerates into a thin filament). This creates constraints for the booming sea cucumber aquaculture industry that would prefer year-round growth (Han et al., 2016). The metabolic regulation of estivation in this species is being studied and DNA methylation has already emerged as a potential contributing mechanism. One study focused on the cyclin B cell cycle regulator (Zhu et al., 2016). Cyclins control the transition between cell cycle stages and, in particular, cyclin B increases during S-phase and regulates the G2/M transition (Ito, 2000). Regulating the cyclin B gene could be crucial to limiting cell cycle activity under hypometabolic conditions, and this is in fact the case – sea cucumber cyclin B expression drops during estivation (Zhu et al., 2016). Interestingly, when DNA methylation was measured at the promoter of the cyclin B gene, it was found that methylation actually decreased in estivating animals. However, the authors suggest that the position of DNA methylation sites may actually regulate binding to a cell cycle gene homology region (CHR) in the promoter which is responsible for transcription of many late cell cycle genes including cyclin B (Müller et al., 2014).

Whereas epigenetics is often discussed in the context of long-time

frames, over the course of development and differentiation of an organism or even transgenerationally, the studies discussed here demonstrate the potential for these mechanisms to also contribute to short-term responses to changing environmental conditions. Most of these studies look at global levels of epigenetic marks across an entire genome and thus do not give an indication as to which specific genes are being regulated. However, the magnitude of the changes that epigenetic marks undergo in relatively short time frames shows that cells are using significant resources for these processes and therefore suggests their importance. More in-depth studies are thus warranted, investigating these mechanisms at the individual gene level which would explain how epigenetic mechanisms may be enabling or fine-tuning adaptations to environmental stress.

2.2. MicroRNA

MiRNA is a class of short non-coding RNA that acts to silence mRNA translation. MiRNAs are typically ~20–22 nucleotides in length and bind to the 3`-untranslated region (UTR) of mRNA. Depending on the degree of complementarity, this leads to either cleavage of the mRNA (in the case of full complementarity) or translational repression and storage of mRNA transcripts into cellular compartments such as P-bodies (He and Hannon, 2004; Liu et al., 2005) (Fig. 2). Hence, miRNA can effectively short-circuit the high energy costs of protein synthesis when cells are stressed and yet retain valuable transcripts until stress is removed and translation can resume.

Like mRNA, miRNA are transcribed by the RNA Pol II-associated transcriptional machinery (Lee et al., 2004), and genes containing miRNA are subject to epigenetic regulation (Ozsolak et al., 2008). Most miRNA coding regions are within introns of coding and non-coding genes, and while they can share the promotor of the outer gene, they often have multiple TSS and therefore separate promoters (Ozsolak et al., 2008). MiRNAs are initially transcribed in an immature form known as a primary miRNA (pri-miRNA) (Lee et al., 2002) (Fig. 2). These structures have a stem-loop of dsRNA containing what will be the mature sequence, as well as 5' and 3' ssRNA tails. The nuclear ribonuclease Drosha, and its cofactor DGCR8, cleave the pri-miRNA approximately 20–22 bp away from the terminal loop of the hairpin to form a structure known as a precursor miRNA (pre-miRNA) (Han et al., 2004). The pre-miRNA is then translocated out of the nucleus by exportin 5 and RanGTP (Bohnsack et al., 2004). In the cytoplasm a second ribonuclease, Dicer, cleaves the terminal loop from the hairpin to create a 20–22 bp dsRNA molecule (Ketting et al., 2001). This pre-miRNA duplex contains a "guide" strand, which is fully or partially complementary to the target mRNA, and a "passenger" strand which is generally not biologically active. One of four members of the Argonaut (AGO) family, with the help of the HSC70-HSP90 chaperone, loads the pre-miRNA duplex, discards the passenger strand and forms the mature RNA-induced silencing complex (RISC) (Iwasaki et al., 2010). Which strand is the guide strand is determined by thermodynamic stability (selecting the strand with the less stable 5' end) and a terminal uracil preference by the AGO protein (Hu et al., 2009; Wu et al., 2009).

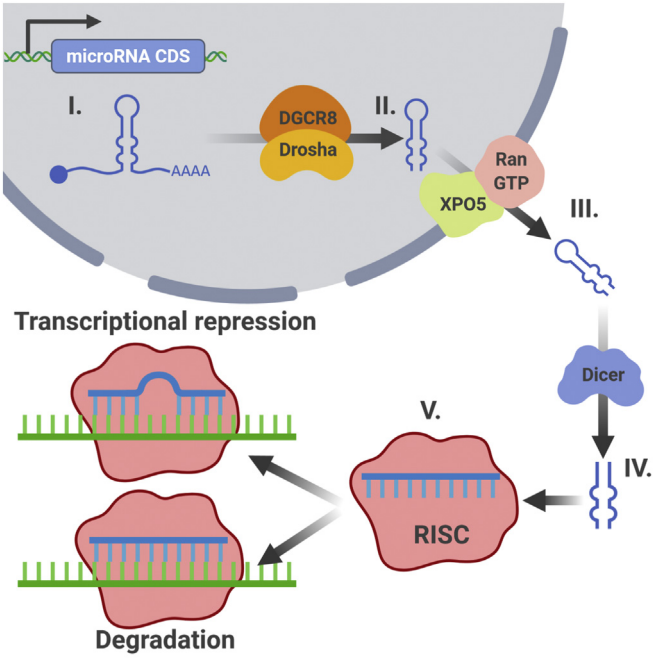

Fig. 2. MicroRNA (miRNA) biogenesis. Maturation of miRNA is a multistep process. (I) Transcription of a primary-miRNA (pri-miRNA) by RNA-Pol II machinery. (II) Drosha and DGCR8 form a ribonuclease complex that cleaves the 5` and 3` ssRNA tails of the pri-miRNA to form a precursor-miRNA (pre-miRNA). (III) Pre-miRNA is exported from the nucleus by the Ran-GTP-dependent exportin 5 (XPO5). (IV) The stem-loop of the pre-miRNA is cleaved by Dicer. (V) Mature miRNA are loaded into a multiprotein RNA-induced silencing complex (RISC), whose main nuclease is a member of the Argonaut family. (VI) The RISC complex cleaves or translationally represses target mRNA depending on the degree of complementarity.

The action of miRNA has been shown to be essential for viability through multiple knockout experiments of miRNA biogenesis proteins (Bernstein et al., 2003; Liu et al., 2004; Wang et al., 2007). Their importance in regulating gene expression is further exemplified by the fact that dysregulation of miRNA is a common factor in cancers and other pathologies (Cheng et al., 2005). Over the past 15 years, studies have suggested that miRNA are a universal regulator of cellular processes. Virtually every cell function or metabolic pathway has been shown to have regulatory inputs from miRNA including, but not limited to, apoptosis (Chang et al., 2007; Cheng et al., 2005; Cimmino et al., 2005), DNA replication (Cannell et al., 2010), the cell cycle (Lal et al., 2009; Lerner et al., 2011; Zhao et al., 2007), differentiation (Chen et al., 2004; Ortega et al., 2010), and myriad metabolic pathways (Fernández-Hernando et al., 2011; Frost and Olson, 2011; Najafi-Shoushtari et al., 2010). MiRNA are a powerful post-transcriptional regulatory mechanism, being metabolically "cheap" to produce, fast-acting, and capable of transcriptome-wide targeting. Thus, it is not surprising that miRNA action has been linked to environmental stress adaptation and hypometabolism.

The roles that miRNA play in adaptations to environmental stresses have been an active area of research by our lab and others in recent years. It has been shown that robust miRNA responses are seen in just about all animals that encounter extreme stress conditions, invertebrates and vertebrates alike. These studies have been well reviewed (Biggar and Storey, 2011, 2015, 2018) and therefore only those in recent years will be discussed here.

The role of miRNA in mammals in multi-day hibernation has been examined in multiple species including 13-lined ground squirrels (Wu et al., 2014), bats (Biggar and Storey, 2014) and the South American marsupial *Dromiciops gliroides* (Hadj-Moussa et al., 2016). MiRNA responses are also part of daily torpor in the grey mouse lemur (*Microcebus murinus*), a small primate from Madagascar. MiRNA action during hibernation/torpor seems to be targeted to well-known attributes of hibernation physiology including the switch from carbohydrate to lipid metabolism, and the use of adipose tissues for lipid storage and thermogenesis during arousal. Studies of mouse lemur torpor also linked MAPK signalling (Biggar et al., 2015), energy signaling (Zhang et al., 2015), and cytoprotection (Wu et al., 2015) to miRNA.

A recent study looked at 122 highly conserved miRNA and used bioinformatic analysis to mine the lemur genome to identify 44 miRNA that had not been identified previously in other species (Biggar et al., 2018). The authors found that 49 (40%) of the measured conserved miRNA and 11 (25%) of the novel miRNA were differentially expressed in the liver of torpid lemurs. After predicting mRNA targets and performing gene set enrichment analysis, several biological processes were

found to be enriched including energy intensive tissue development and cell differentiation. The fact that these results do not exactly align with miRNA results from other hibernators is not surprising given their different physiological traits. While many hibernators use forms of cold-hibernation (body temperature tracking closely with ambient temperature in cold climates), the tropical range of the lemur means that during daily torpor bouts their body temperature only drops a few degrees. Lemurs also do not have to survive on only internal fuel reserves for months at a time, and therefore it is not surprising that miRNA that target energy expensive processes and pathways are differentially regulated, rather than those that would regulate lipid metabolism, for example. This point is exemplified by KEGG pathway analysis which indicated that Focal Adhesion (hsa04510) and Adherens Junction (hsa04520) pathways were significantly targeted by torpor-responsive miRNA. These pathways are directly involved in cell motility, cell proliferation, cell barrier, and growth and differentiation. In each of these pathways, nearly all members were targeted by one or more torpor-responsive miRNA, signifying just how comprehensive miRNA regulation of energy expensive pathways may be. Targeting these energy expensive pathways aligns with the energy conserving function of daily torpor of the lemur.

While freeze tolerance of the wood frog has been well studied in organs such as the liver and skeletal muscle (Storey and Storey, 2017), relatively little is known about how this species maintains neural integrity over multiple freeze-thaw cycles. One approach to answering this problem is by identifying differentially-expressed miRNA and what cellular processes/pathways these could regulate during freeze/thaw. A recent study found that nearly all members of the miRNA biogenesis pathway showed decreased proteins levels in the brain of frozen wood frogs, including Drosha, Dicer, AGO1, and AGO2, indicating potentially lower miRNA biogenesis capacity and RISC activity in the frozen state (Hadj-Moussa and Storey, 2018a). These results were complemented by generally reduced miRNA expression levels in 24 h frozen and/or 8 h thawed frogs. Indeed, of the 113 miRNA measured, 41 showed significantly reduced levels during freezing and/or thawing, whereas only two were upregulated with miR-451-5p being the only one that was specifically upregulated during freezing itself. Interestingly, miR-451 is known to be overexpressed in response to high glucose levels – the condition the wood frog brain is in while frozen (Storey, 1987a) and it contributes to proliferation in glioma cells (Godlewski et al., 2010). Whereas the function of miR-451 remains speculative for wood frog brain, maintenance of cellular proliferation and cell cycling may be necessary to maintain neural connections in the face of freezing damage. The predicted mRNA targets of the downregulated miRNA included those involved in synaptic vesicle signaling (Hadj-Moussa and Storey, 2018a). This indicates the potential for these genes to be upregulated during freezing and signifies that miRNA may play a neurofunctional or neuroprotective role in the brain while frogs are frozen.

Environmental stress responsive miRNA has also been studied in multiple invertebrate lineages. Notable is a study on the Humbolt jumbo squid (*Dosidicus gigas*) in the Pacific Ocean that undergoes a diurnal vertical migration, descending into the oxygen minimum zone during the day, and then rising to the surface at night to feed. While at depth, these animals are exposed to multiple stresses – hypoxia, cold temperature, high pressure – and enter a hypometabolic state to cope with the stressful environment (Rosa and Seibel, 2010). Recently, the first study of miRNA in a cephalopod examined hypoxia-induced miRNA in this species (Hadj-Moussa et al., 2018), measuring 39 conserved invertebrate miRNA in the brain, mantle muscle and brachial heart. Results showed that all differentially expressed miRNA were elevated in both brain and heart tissue under hypoxia and are an interesting contrast to what was reported for wood frog brain. While these two animals experience different stresses (hypoxia, cold, high pressure water vs. freezing temperatures), they share a necessity for neuroprotective measures, and these results show that these responses may be diverse, a situation that may be involved only with deeper studies. A global increase in miRNA suggests translational repression, which may be necessary for the brain to conserve energy in the oxygen poor environment. However, specific miRNA have been shown to enhance neural outgrowth, such as miR-133 (Xin et al., 2012). This miRNA was also upregulated in the heart and along with miR-2a-3p miR-2c/d-3p that have anti-apoptotic functions (Li et al., 2015; Zhang and Cohen, 2013). Anti-apoptotic mechanisms have been explored in other animals that tolerate environmental stresses that would otherwise cause cell death including freeze tolerant wood frogs, and hibernating ground squirrels (Gerber et al., 2016; Rouble et al., 2013). The fact that anti-apoptosis is a common finding in these animals shows the importance of preserving cellular and tissue integrity in the face of stressful conditions. While this was the first investigation of miRNA in a cephalopod, no doubt there will continue to be interesting findings in these animals given their unique RNA mechanisms (Liscovitch-Brauer et al., 2017).

Gastropod molluscs also use metabolic rate depression to deal with environmental stresses, which is the case for the milk snail (*Otala lactea*), that estivates when water is scarce (Herreid, 1977). When miRNA were examined in 10-day estivating snails, miRNA upregulated in foot muscle were related to anti-apoptotic functions similar to results from other animals previously discussed (Hoyeck et al., 2019), again emphasizing the importance of maintaining cell/tissue integrity and viability while torpid by limiting or preventing apoptosis.

Another aquatic invertebrate able to endure little to no oxygen is the Northern crayfish (*Orconectes virilis*), that can encounter oxygen limitation under various circumstances, e.g. in stagnant summer waters, or during the winter when water is ice-locked. To add to our understanding of anoxia tolerance in this animal, a study examined expression of 76 miRNA in crayfish after short (2 h) or long (20 h) exposure to anoxic water (English et al., 2018). Tail muscle and hepatopancreas showed distinct differences in their responses, with only four miRNA differentially expressed in muscle but 22 in the hepatopancreas. This may reflect substantial differences in roles played by these tissues under anoxic conditions. The hepatopancreas is a more metabolically active tissue, thus under anoxic conditions it appears to rely more on miRNA mediated metabolic regulation than does tail muscle. Also contrasting were the differences in 2 h and 20 h anoxia exposures. In both tissues, fewer miRNA were differentially regulated in the longer exposure than the shorter. It was hypothesized that anoxia-responsive reorganization of metabolism, cellular processes, and physiology is more active in a short-term exposure period (2 h), thus using miRNA more intensely, than after prolonged exposure (20 h). In the longer-term, gene expression patterns settle into a state conducive for long-term anoxia exposure and thus fewer changes to miRNA expression may be needed. Also of note was the regulation of miRNA linked with the hypoxia-inducible transcription factor (HIF1α) (English et al., 2018). MiR-133-3p, miR-33-5p, miR-125-5p, and miR-190-5p all contribute to HIF1α regulation either directly or indirectly, and thus the changes in expression of these miRNA may contribute to the post-transcriptional regulation of HIF1α activity at to help this species survive prolonged anoxia. While HIF genes are well conserved in anoxia tolerant and intolerant species alike, the use of additional HIF1α regulatory mechanisms, such as miRNA, in this species and others may contribute to their remarkable anoxia tolerance.

Without fail, studies examining miRNA in the context of environmental stress adaptation show the importance of these small molecules. Studies to date have taken a targeted approach, but with the falling costs of sequencing technologies it will not be long before sequencing the entire miRNAomes of animals will be commonplace.

3. Emerging technologies and their implications

3.1. Genome editing

In animals, particularly mammals, genome editing typically involves the use of a two-part system – one part containing a means of

recognizing a sequence of interest, and one part with the ability to act on that sequence (usually inducing a double strand break with a nuclease). Previously, the best systems for genome editing, zinc-finger nucleases and TALENs, used protein-based components for both recognition and action. This meant that editing a single genomic locus involved designing, synthesizing, and refining a polypeptide sequence that would recognize a nucleic acid sequence, which is laborious and inexact (Dahlem et al., 2012). The discovery of CRISPR-Cas9 alleviated some of these difficulties by introducing a system that used a nucleotide sequence for recognition and targeting, and therefore could be easily designed and synthesized for a fraction of the cost (Jinek et al., 2012). In the past, the combination of species-specific resources (genomic sequences, efficient husbandry protocols, species-specific reagents, antibodies, etc.) and high technical and monetary cost meant that genome editing was used with only the most developed model organisms. Reducing the technical and cost barriers of genome editing means that a more diverse range of organisms can be investigated using these powerful tools. It no longer seems implausible to deploy genome editing tools in models of environmental stress adaptation such as hibernating ground squirrels and freeze tolerant wood frogs since well developed genome editing paradigms are present in closely related species (Platt et al., 2014; Wang et al., 2015a).

Genome editing tools could allow outstanding questions about environmental stress adaptation to be answered and new questions to be asked. For example, a hibernation phenotype is present among most mammalian lineages and thus the debate as to whether it is a plesiomorphic trait (first developed in an ancestral species and modified or lost in subsequent lineages) is inconclusive (Lovegrove, 2012). If hibernation was an ancestral trait to all mammalian lineages, then the expression of this phenotype in non-hibernators (e.g. humans) may not be as difficult as intuitively thought. The use of genome editing tools could accelerate the determination of just which genes are necessary for hibernation. Currently, studies investigating genes important for hibernation are generally limited to correlation analysis (e.g. differential gene expression and metabolites during hibernation) and comparison to non-hibernators (e.g. differences in gene sequences, gene expression in response to cold temperatures etc.), but direct manipulation of candidate genes will help determine their causal role. A systematic replacement of genes thought to be important to hibernation with their counterparts from non-hibernators could determine which genes are in fact necessary. An analogous approach could be taken with non-hibernators, such as mice or rats, where gene variants from hibernators would be introduced into these animals. While introduction of a small number of hibernator gene variants into non-hibernators likely would not result in induction of a full hibernation phenotype, a small number of genes may be found to improve parameters of interest such as organ and tissue resistance to cold temperatures or ischemia-reperfusion tolerance, and therefore could have important biomedical applications.

While the CRIPSR-Cas9 system is used to remove or edit genes, variants of Cas9 with no-nuclease activity, known as dCas9 (dead Cas9), have been developed. These systems use the same specific targeting mechanisms, but replace nuclease activity with other functional units such as transcriptional activators, epigenetic modifiers, fluorescent proteins, etc. (Xu and Qi, 2019). Targeting the genome specifically with a variety of programmable functions allows not only for the investigation of molecular and regulatory mechanisms, but also how these mechanisms are used to create remarkable phenotypes such as those seen in animals adapted to extreme environmental conditions. Indeed, a dCas9 protein with a DNMT attached was recently used to demonstrate gene expression silencing by DNA methylation in a causative manner (Vojta et al., 2016), and a programmable DNA demethylation system was created with a dCas9 TET1 fusion (Morita et al., 2016). Both systems take advantage of the CRISPR-dCas9 targeting system where targets are defined by supplying one or more nucleotide sequences complementary to the region or regions of interest. Together these allow for fined-tuned control over DNA methylation patterns that could be used to further our understanding of the role of DNA methylation in processes such as anti-atrophy in ground squirrels. As previously discussed, hibernators show remarkable resistance to muscle atrophy when hibernating and DNA methylation of the *mef2c* gene may be implicated (Alvarado et al., 2015). Once a CRISPR-Cas9 protocol is established in the 13-lined ground squirrel, incorporation of inducible and programmable DNA methylation machinery could be possible (Xu and Qi, 2019), allowing for investigation of DNA methylation of *mef2c* and other genes in a causal manner. If regulation of genes such as *mef2c* by DNA methylation contributes meaningfully to anti-atrophy, the use of the same programmable DNA methylation machinery could be used to produce these effects in non-hibernators and may eventually be used as a therapeutic.

CRISPR-like systems are still in their infancy. Concerns over off-target effects and efficiencies are well founded (Wang et al., 2015c) but advances in our understanding of how target sequences should be designed (Dang et al., 2015), discovery of alternative Cas proteins (Murugan et al., 2017), and using directed evolution to increase the specificity of existing systems (Lee et al., 2018) will only improve and expand the use of genome editing in biological research.

3.2. Novel sequencing applications

As predicted, the falling costs of genome sequencing has led to the ubiquitous use of next generation sequencing technologies. The two first applications of these technologies were to sequence the genome and sequence and quantify the transcriptome of myriad species. The types of information that can be examined, however, now extends far beyond these goals. Enhanced data analysis and/or sample preparation techniques allow for the measurement of everything from specific classes of RNA molecules to chromosomal organization to sequencing the epigenome and more (Stuart and Satija, 2019).

The organization and higher order structures of chromatin confer crucial regulatory mechanisms of gene expression. Cis-regulatory elements like silencers and enhancers can interact directly or indirectly with promoters to repress or activate transcription. Similarly, chromatin can be in different conformational states that allow or disallow interactions with transcriptional machinery. Therefore, a complete understanding of gene regulation requires more information on chromosome interactions and structures and thus this is an important, but unexplored, area of study in the field of environmental stress adaptations. Low-throughput methods for investigating chromatin interactions have existed for many years (Dekker et al., 2002). Known as chromatin conformation capture, this technique involves crosslinking interacting loci of the genome, restriction digesting the genome, then ligating the crosslinked strands together so that interaction frequency could subsequently be measured by PCR techniques. Sequencing technologies have been applied to this method to extend it from investigating interactions of two pre-determined loci in the genome, to quantifying interactions of every locus in the genome with every other locus, known as Hi-C (Belton et al., 2012).

Techniques for investigating chromosome conformation, such as Hi-C, may give novel insights for animals that undergo changes in physiological, metabolic or gene expression state in response to specific environmental conditions. The freeze tolerant wood frog, for example, undergoes profound metabolic rate depression involving cessation of nearly all gross physiological functions when entering a frozen state (Layne et al., 1989). The frozen state is qualitatively different from the unfrozen state, involving an apparent reorganization of gene expression programs, where only those genes necessary for freezing survival are expressed. Is this change in gene expression a result of chromatin reorganization? No studies have directly addressed this question, but results from epigenetic studies are consistent with this possibility (Hawkins and Storey, 2018; Zhang et al., 2019). DNA methylation and histone modifications are tightly associated with chromatin conformations. For example, DNA methylation and the histone modification

H3K9me3 are necessary for recruiting the heterochromatin protein HP-1 (Fuks, 2005) which is responsible for transcriptionally silent heterochromatin formation. In wood frog muscle, global DNA methylation increased over two-fold in the frozen state, which could have potentially profound implications for chromatin organization. Use of a technique such as Hi-C could determine whether macro-scale changes to chromatin organization are occurring in the wood frog to switch from an unfrozen to frozen gene expression program. Similarly, major changes in gene expression occur in animals such as the anoxia tolerant turtle and in mammalian hibernators. These animals are perfect platforms for studying gene expression plasticity, and the establishment of transcriptional states necessary for surviving particular environments.

With respect to the context of this review, the animal most studied in terms of epigenetics is the 13-lined ground squirrel (Table 1). Ultimately, epigenetic mechanisms act at the gene level, and thus the fact that DNA methylation of the promoter of only one gene in the 13-lined ground squirrel has been studied means that there is still a trove of information to be gained from the epigenome of this species. Sequencing techniques can be used to study the full epigenome and how it changes in response to environmental conditions. Bisulfite-sequencing is a method for chemically modifying unmethylated cytosines to form uracils across the genome; uracils can then be detected by sequencing and thereby provide a measure of DNA methylation at the base-pair level (Urich et al., 2015). For histone modifications, chromatin immunoprecipitation sequencing (ChIP-seq) is used to sequence DNA loci enriched for histone modifications of choice. First, proteins (including histones) are crosslinked to the DNA which is then sheared by sonication. An antibody specific for the targeted histone modification is used to enrich DNA fragments crosslinked to the modified histones, and then after the enriched DNA fragments are purified, they are sequenced and histone modifications at genomic loci can be quantified. The combination of ChIP-seq and bisulfite-seq would allow epigenomic profiling of multiple stress-tolerant animals of interest and determine the epigenetic contributions to various environmental adaptations. For example, the anoxia tolerant turtle showed 30% and 50% increases in global DNA methylation in liver and white muscle after anoxia exposure (Wijenayake and Storey, 2016). How these large increases in DNA methylation are distributed across the genome is not yet known, although, given the magnitude of change the use of methods like bisulfite-sequencing may elucidate previously unknown gene regulatory patterns with respect to anoxia tolerance.

The number of sequencing resources available to comparative biochemists will also show a step change increase with the development and refinement of the latest generation of sequencing technologies. Nanopore sequencing technology is one such advance. This method differs from current sequencing-by-synthesis technologies in multiple respects, and functions fundamentally by threading DNA molecules (or other biopolymers) through a nanopore protein separating two compartments and measuring changes in ionic current. The magnitude of change in ionic current depends on the molecule blocking the pore opening and this can be used to differentiate nucleobases as they pass through the nanopore (Branton et al., 2008). Nanopore based technologies have been commercially available for a few years (Jain et al., 2016), but widespread replacement of more expensive sequencing-by-synthesis technologies has not yet occurred, most likely due to the lagging performance of current nanopore solutions. With the maturation of nanopore performance, nanopore sequencing technologies may be common in laboratories, especially due to their versatility and minimal equipment and reagent requirements. For example, the Oxford Nanopore minION weighs just 90 g and has been used to sequence samples in the field by teams responding to the Ebola crisis in West Africa (Quick et al., 2016), and on the International Space Station (Castro-Wallace et al., 2017). Therefore, this technology is perfectly suited for sequencing uncommonly studied animals in a variety of environmental conditions. For example, to perform a comparative transcriptomic study of multiple populations of a given species spanning a variety of environmental conditions (e.g. altitudes, temperatures, water salinities etc.), researchers would currently have to store samples cryogenically until returning to a laboratory or would have to perform RNA extraction and cDNA synthesis in field using specialized reagents and equipment. Nanopore sequencing technologies are not only portable enough to fit in a backpack, but can also sequence RNA directly (Garalde et al., 2018), thus making in-field sequencing a reality.

3.3. Single cell analysis and multiplexed localization

Adaptations to environmental stresses have tissue- and organ-specific components and thus these are the most common level of biological organization studied with regard to these adaptations. While gross function can be learned from this strategy, a significant amount of information is lost by not studying the functions of specific cell types, particularly in heterogenous tissues. While there are methods for enriching, separating, or examining specific populations of cells based on predetermined criteria (e.g. flow cytometry-based methods), new single cell analysis methods are being developed which allow researchers to examine all cells from a sample individually.

Single-cell RNA-seq (scRNA-seq) is a method of sequencing the transcriptome of individual cells from a sample. Recent iterations of this method can perform RNA-seq on millions of cells in parallel. This is accomplished by dissociating cells, then performing iterative barcoding such that all the transcripts from a single cell have a common barcode that differentiate them from all other cells, allowing transcripts to be pooled from all cells prior to sequencing (Rosenberg et al., 2018). A bioinformatic dissection of the tissue can then be performed by clustering cells based on similar transcriptomes, with results that closely match canonically defined cell types (Stuart and Satija, 2019).

Similarly, novel sequencing applications discussed previously (and others) are being adapted to single cell formats. Chromosome conformation, genomes, epigenomes, transcriptomes, and spatial information can all be measured individually, and often have been combined into multi-modal methods for extremely powerful analytic capabilities (Stuart and Satija, 2019). For example, the simultaneous quantification of mRNA transcripts and chromatin accessibility using sci-CAR can be used to match cis-regulatory elements to the genes they regulate by analysing the covariation of their accessibility and transcript abundance throughout the cell population (Cao et al., 2018). Likewise, non-sequencing techniques are also being developed. Moffitt et al. (2018) have demonstrated a highly multiplexed fluorescent in situ hybridization (FISH) method for visualizing > 100 transcripts in tissue sections containing hundreds of thousands of cells. Similarly, MALDI mass spectrometry imaging performed on a series of tissue slices was used to show the three-dimension localization of specific lipids at the site of spinal cord injury (Quanico et al., 2018). Techniques such as these open possibilities of research not available to previous biochemical and molecular biology researchers.

The implications of these technologies for studying environmental stress adaptation cannot be overstated. The freeze-tolerant wood frog, for example, possesses unique glucose regulation that allows the mobilization of massive amounts of glucose from the liver to the blood for transport around the body. Currently, all characterization of the liver has been at the whole-organ level and thus the contributions to the freeze-tolerant hyperglycemic phenotypes of other cell types in the liver is certainly masked by the abundance of hepatocytes. Likewise, although insulin from the wood frog has a unique structure (Conlon et al., 1998), almost nothing is known about the responses of the pancreas during freeze-thaw cycles, which may partially be due to its tiny size and the potentially confounding data that would result from analysing it as a whole. Understanding how the distinct cell types of both of these heterogeneous tissues are regulated and respond during freezing would benefit our understanding of freeze tolerance as a whole – a task that could be accomplished using single cell analysis techniques.

These techniques could be used to answer myriad questions about a

variety of animals discussed in this review. Hibernators brown adipose is crucial for maintaining a minimal body temperature set point and for powering rewarming upon arousal. There is evidence that this non-shivering thermogenesis is influenced by tissue macrophages (Wolf et al., 2017), which could not be easily examined from a tissue-level analysis, but could be elucidated with techniques such as scRNA-seq. Likewise, the complex adaptations supporting prolonged anoxia tolerance of turtles could be investigated at the single cell level, particularly in the brain where there is considerable heterogeneity even within cell types. Several neurotransmitters and metabolites have been implicated in anoxia tolerance as a means to either suppress energy expenditure or maintain some basal level of function to preserve neural integrity (Lutz and Milton, 2004; Miles et al., 2018). Multiplexed localization using methods such as MALDI-imaging could show the origin and distribution of many neuroactive compounds across the whole brain that would give insight into how these animals resist significant damage in the face of prolonged anoxia.

The development of new technologies continues to bring us closer to a complete understanding of animal adaptations to environmental stress. With each innovation, new functions of cells can be explored. Whereas applications of these technologies to traditional model organisms is necessary to elucidate the prototypical biological paradigms, applying the same technologies to diverse species under diverse conditions will identify the full range of biological possibilities.

4. Use of high-throughput technologies

Prior to the genomic era, there was a sense that most questions about the cell, tissues, and whole organisms could be answered by sequencing and analysing the genome. Now that we have entered the post-genome era, it is clear that possessing the genomic sequence of an organism only gets you so far and that many more “unknown unknowns” are probable (Logan, 2009). Advances in next-generation sequencing (NGS) and mass spectrometry technologies enable high-throughput examination of biomolecules at a price that is now becoming accessible to many researchers. The widespread use of these technologies will allow for the identification of novel and common mechanisms in animals that are adapted to deal with diverse environmental stresses. In this section, we discuss studies that have harnessed these technologies to give insights into environmental stress adaptations (Table 2).

4.1. Genomics

Whereas the genome alone cannot resolve all questions about the functions of the cell, it does give important information about species-specific genes and genomic features that allow incredible adaptations to environmental stresses and next generation sequencing is revealing new possibilities. An example of this is tardigrades which are aquatic microfauna that have incredible tolerance to desiccation. While desiccated these animals enter a state of anhydrobiosis characterized by a lack of metabolic activity (Wełnicz et al., 2011); some species are able to survive temperature changes from close to absolute zero to near boiling, exposure to high radiation levels, and even exposure to the vacuum of space (Hashimoto et al., 2016). Initial genomic sequencing of tardigrades elicited controversy (Bemm et al., 2016), spawning a recent high quality sequencing effort (Hashimoto et al., 2016). Results from this study showed that the tardigrade species *Ramazzottius varieornatus* not only has an extended set of antioxidant-related enzymes, but also a selective loss of canonical stress responsive pathways. This species also showed 16 superoxide dismutase (SOD) genes which may provide superior tolerance to oxidative damage during desiccation. This species also possesses the bacterial-derived clade II catalase that can maintain activity under otherwise denaturing conditions. Interestingly, *R. varieornatus* is missing pathways involved in mTOR signalling, including HIF1α, which the authors postulate would decrease autophagy and promote the resumption of cellular activity upon rehydration. It goes without saying that further analysis of the genome of this species will reveal potentially novel genomic features that are conducive to surviving truly extreme conditions.

Similar to tardigrades, some species of nematodes are also highly resistant to environmental stresses including desiccation and freezing. The nematode *Panagrolaimus* sp. DAW1 is found in the Antarctic and is the only animal species known to survive intracellular ice formation in most or all cells of its body (Wharton and Ferns, 1995). Other freeze tolerant animals endure only extracellular freezing while employing cryoprotective measures to maintain the cytoplasm in a liquid state. Sequencing of the genome of this nematode indicated that one potential factor that may support intracellular freezing survival is the occurrence of duplicated trehalose-6-synthase genes (Thorne et al., 2014). Trehalose production as a protectant and duplication of related genes has been found in other species of nematodes that survive desiccation and trehalose is also a known cryoprotectant in other invertebrates (Hino et al., 1990). Trehalose action is particularly effective at stabilizing the bilayer structure of membranes (Crowe, 2015) and, hence, duplication of trehalose-related genes may provide the enhanced capacity to synthesize trehalose that may be required by very small animals experiencing rapid dehydration. Also found in the genome were nine members of the late embryogenesis abundant (LEA) protein family. This family of proteins is implicated in desiccation tolerance of plants and animals (Liu et al., 2019) and have multiple protective functions including prevention of protein aggregation (Goyal et al., 2005) due to their intrinsically disordered structures (Chakrabortee et al., 2012). The exact function of these genes in nematodes during freezing has yet to be elucidated, but, the presence of nine LEA genes in the genome suggests their importance to this remarkably resilient species.

A comparative analysis of the genomes of multiple mammals with distinctive phenotypes was recently performed to identify genomic regions that may be responsible for these phenotypes (Ferris et al., 2018). The mammals of interest included elephants for their large body size and resistance to DNA damage, cetaceans for their adaptations to fully aquatic environments, and hibernating squirrels and bats, among others. The authors compared conserved regions of the genomes of these mammals to the genomes of many other mammals to find evolutionarily accelerated regions (ARs) specific to the species of interest. These ARs are regions of the genome that are typically highly conserved across species, but are found with increased mutation rates in a specific species. They authors postulated that these ARs would only exist if there are major phenotypic effects that benefit that specific species. For example, ARs found only in hibernating animals are likely to be necessary for their hibernation phenotype. For all species of interest, it was found that the clear majority (65–82%) of ARs were found in areas that correspond to non-coding elements of the human genome, suggesting that these amazing phenotypes arise less from changes in the sequences of genes and their protein products themselves and more from altered regulation of gene expression. Indeed, when comparing just the hibernating mammals, ARs were highly enriched in regions of the genome associated with the presence of methylated histones (as determined from human ChIP-seq experiments), further demonstrating the importance of histone modification and epigenetics to the hibernator phenotype. As for specific genes enriched for ARs, the forkhead box protein P1 (FOXP1) was highlighted as a gene enriched for ARs in both hibernating squirrels and bats, which may suggest that the function of this transcription factor is modified to contribute to their common hibernation phenotype.

Comparative genomic studies such as this one continue to highlight the importance of modifications to genes and to areas of the genome that are similar in related species to produce vastly different phenotypes. Searching for novel adaptation-specific genes is important but examining the nuances of slight genomic differences may also be a fruitful strategy.

Table 2
Summary of omics studies in animals adapted to environmental stresses.

Adaptation	Species	Common name	Omics method	Tissue	Results	Reference
Dessication	*Ramazzottius varieornatus*	Tardigrade, water bears	Genomics	-	16 SOD genes for enhanced oxidative damage tolerance, clade II catalase to maintain antioxidation activity under denaturing conditions, loss of mTOR pathway and HIF1α may decrease autophagy	Hashimoto et al. (2016)
Freeze tolerance	*Panagrolaimus* sp. DAW1	Antarctic nematode	Genomics	-	Duplicate trehalose-6-synthase genes may enhance cryoprotectant production. Nine LEA genes increase resistance to protein aggregation and are found in desiccation tolerance plants and animals	Thorne et al. (2014)
			Transcriptomics	Whole organism	Collagen genes upregulation at onset of freezing would play a role in water distribution. Genes regulating body morphogenesis, carbohydrate metabolism, and dauer regulated during freezing. STRING analysis showed shrinking gene interaction networks during freezing, network growth after thawing	Thorne et al. (2017)
	Rana sylvatica	Wood frog	Phosphoproteomics	Liver	Phosphorylation of PFKFB2 and glycogenolytic enzymes support export of glucose during freezing, but catabolism glucose during anoxia and dehydration exposure.	Hawkins et al. (2019)
Dehydration tolerance	*Rana sylvatica*					
Anoxia tolerance	*Rana sylvatica*					
Hibernation	*Myotis lucifugus*	Little brown bat	Genomics	-	Hibernators share high number of ARs, particularly in non-coding regions and regions highly methylated suggesting these regions have common contribution to hibernation. Hibernators shared FOXP1	Ferris et al. (2018)
	Eptesicus fuscus	Big brown bat	Genomics	-		
	Ictidomys tridecemlineatus	13-lined ground squirrel	Genomics	-		
			Transcriptomics	White adipose tissue	Upregulation of lipogenesis genes prior to hibernation, albumin upregulation directly prior to hibernation as potential preparatory mechanism for lipid distribution during hibernation	Hampton et al. (2011)
			Proteomics	Skeletal muscle	Levels of proteins involved in fatty acid metabolism rose as hibernation approached, the opposite trend seen in carbohydrate related proteins. At end of hibernation, fast switch in proteins levels of carbohydrate related and fatty acid related proteins	Anderson et al. (2016)
			Proteomics, phosphoproteomics	Brown adipose tissue	Levels of most mitochondrial proteins showed no change. Phosphorylation of HSL and PDH changed during hibernation	Vertommen et al. (2017)
	Spermophilus parryii	Arctic ground squirrel	Transcriptomics	Liver	Glycolytic and fatty acid synthesis genes downregulated while fatty acid catabolism genes upregulated during hibernation	Yan et al. (2008)
	Myotis brandtii	Brandt's bat	Transcriptomics	Liver	Downregulation of glycolytic gene sets with upregulation of lipid metabolism during long-term hibernation	Seim et al. (2013)
	Ursus americanus	American black bear	Transcriptomics	Liver	Fatty acid oxidation gene upregulation and downregulation of amino acid catabolism and cellular respiration genes during hibernation	Fedorov et al. (2011)
			Transcriptomics	Kidney	Anti-inflammatory cytokine supressing genes such as SOCS2, CISH, and SERPINC1 upregulated post-hibernation with no increases in damage markers suggesting protective function	Srivastava et al. (2019)
	Ursus arctos	Brown bear	Proteomics	Skeletal muscle	Unexpected increase in glycolytic proteins and decrease in lipid oxidation proteins. Downregulation of mitochondrial respiration proteins. Increased plasma fatty acids and ketones suggest lipids are still main fuel source	Chazarin et al. (2019)
	Dromiciops gliroides	Monito del monte	Transcriptomics	Brain, liver, skeletal muscle	Increased expression of PDK4 to switch from carbohydrate to lipid metabolism in the brain. High TXNIP expression in multiple tissues which may promote fatty acid oxidation and inhibit glucose uptake	Nespolo et al. (2018)
	Cheirogalaus crossleyi	Furry-eared dwarf lemur	Transcriptomics	White adipose tissue	PDK4 strongly increased with decrease in PDH complex members may contribute to switch from carbohydrate to lipid metabolism. Lipid catabolism genes upregulated and lipid biosynthesis genes downregulated	Faherty et al. (2018)
	Pogona vitticeps	Australian central bearded dragon	Transcriptomics	Brain, heart, skeletal muscle	Genes part of Sin31 HDAC complex, SWI/SNF complex, ATAC complex, and polycomb-group were differentially expressed. Genes indicating negative regulation of cell cycle during hibernation, and upregulation of hypoxia related genes. Genes related to lipid and carbohydrate catabolism were downregulated whereas gluconeogenic genes were upregulated	Capraro et al. (2019)

Abbreviations: SOD – Superoxide dismutase; mTOR – Mammalian target of rapamycin; HIF1α – Hypoxia inducible factor 1 alpha; LEA – Late embryogenesis abundant; AR – Evolutionarily accelerated region; FOXP1 – forkhead box protein P1; PDK4 – pyruvate dehydrogenase kinase 4; TXNIP – thioredoxin-interacting protein; PDK – Pyruvate dehydrogenase; HDAC – Histone deacetylase; SWI/SNF – SWItch/Sucrose Non-Fermentable; ATAC – Ada2a containing complex; HSL – Hormone sensitive lipase.

4.2. Transcriptomics

Gene expression profiles can identify genes and pathways involved in the response to various environmental stresses. While there is often some discordance between transcript and protein expression profiles, a transcriptomic analysis is a powerful tool for the study of environmental stress adaptation, especially now that such analyses have a relatively low price point and easy-to-use accessible software tools have simplified data analysis. Analytical methods such as RNA-seq are more and more becoming the go-to genetics approach to studying stress-responsive adaptation.

For example, several transcriptomics studies have been conducted on hibernating mammals (Table 2). Common to most hibernators is the switch from sourcing carbohydrate fuels to a primary reliance on lipids during seasonal hibernation. Therefore, it is not surprising that evidence of this metabolic shift is present in most transcriptomics studies of mammalian hibernators. For example, in the Arctic ground squirrel, *Spermophilus parryii*, genes coding for glycolytic enzymes (glucokinase in particular) showed downregulation during torpor, whereas fatty acid catabolism genes were upregulated and fatty acid synthesis genes were strongly downregulated in liver (Yan et al., 2008). Similarly, analysis of 13-lined ground squirrels revealed upregulation of lipogenesis related genes in WAT in animals sampled during August and October (Hampton et al., 2011). In these samples both fatty acid binding protein 4 (FABP4) and leptin transcripts were overexpressed, both of which are markers of lipid accumulation and increased fat mass. The samples from October animals also showed a marked increase in albumin transcripts, that the authors postulated was a preparatory measure for the upcoming need to distribute lipids from white adipose via the blood to be used as fuel during hibernation.

Other hibernators also showed transcriptomic evidence of metabolic shifts either seasonally or over torpor/arousal. For example, gene set enrichment analysis of RNA-seq data from the liver of control and long-term hibernating Brandt's bat, *Myotis brandtii*, found downregulation of transcripts associated with glycolysis and upregulation of lipid metabolism (Seim et al., 2013). A cDNA microarray analysis of the American black bear, *Ursus americanus*, comparing the liver of summer active and winter hibernating bears showed fatty-acid β-oxidation was upregulated whereas amino acid catabolism and cellular respiration downregulated, again consistent with a heavy reliance on lipids as a fuel source during hibernation (Fedorov et al., 2011).

The transcriptional evidence for a metabolic switch extends across the phylogenetic tree of hibernating mammals extending both to a South American hibernating marsupial, *D. gliroides* (Nespolo et al., 2018) and a hibernating lemur *Cheirogalaus crossleyi* (Faherty et al., 2018) (Table 2). Interestingly, transcriptomic analysis of *D. gliroides* showed increased pyruvate dehydrogenase kinase 4 (PDK4) expression in the brain during torpor (Nespolo et al., 2018). PDK4 regulates the pyruvate dehydrogenase complex (PDH) via reversible phosphorylation of the enzyme and is crucial to the switch from carbohydrate to lipid catabolism as a primary fuel source during torpor. PDK4 upregulation and/or increased phosphorylation of PDH has been shown during hibernation in *D. gliroides* and 13-lined ground squirrels (Wijenayake et al., 2017, 2018b). Another potential mechanism contributing to the fuel switch is suggested by the increase of thioredoxin-interacting protein (TXNIP) seen in the brain, liver, and skeletal muscle of *D. gliroides* during torpor. TXNIP is a regulator of thioredoxin, therefore affecting redox activities. The protein also has hibernation relevant functions in promoting fatty acid oxidation and inhibiting uptake of glucose (Nespolo et al., 2018). Similarly, transcriptional evidence of PDK4 activation was also found in the lemur *C. crossleyi* (Faherty et al., 2018). This study used the ecology method of capture-mark-recapture to study the same animals over the course of a hibernation cycle and performed RNA-seq on biopsy samples of WAT. PDK4 was strongly increased in the lemurs during torpor, consistent with a decrease in members of the PDH complex. Other lipid metabolism genes were upregulated during torpor including phospholipid phosphatase 1 and apolipoprotein C-II, whereas lipid biosynthesis appears to be transcriptionally repressed as the expression of multiple genes was decreased (Faherty et al., 2018). Although the genes and tissue patterns of expression vary in each of these studies, the fact that transcriptomic evidence for the switch from carbohydrate to lipid metabolism is present in each of these animals gives further evidence for the utility of transcriptomics.

4.3. Proteomics

Mass spectrometry has long been used to identify and quantify proteins, peptides, and other biomolecules. Increases in instrument resolution, technique development, software tools, and databases means that the use of mass spectrometry for high-throughput analysis of a sample is accessible to non-specialists and can be used as a tool for environmental stress adaptation research (Hu et al., 2016). As discussed, genomics and transcriptomics can give insight into the possible mechanisms and responses to stresses, whereas proteomics allows investigation of the actual actors carrying out cell functions. Proteomics methods are not without their flaws. Unlike transcriptomic or genomic techniques that can amplify starting materials using PCR, proteomics datasets are generally skewed to high abundance proteins. Potentially important, but low abundance proteins and peptides may be masked by others; a notorious problem in plasma proteomics for example were just ten proteins account for > 90% of plasma protein content (Millioni et al., 2011). Likewise, species specific mass spectrometry databases are crucial to efficient and accurate analysis of mass spectrums, the lack of which for newly studied species can hamper identification of proteins. While these issues present some limitations for mass spectrometry-based proteomics, studies that employ these methods continue to elicit new and novel insights into diverse animals and their adaptations, whereas further development and application of targeted proteomics approaches, such as selected reaction monitoring and multiple reaction monitoring, will aid in quantitation. Here we look at the use of quadrupole-based mass spectrometry for high-throughput study of the proteome responses during environmental stress adaptation (Table 2).

Proteomics has been a fruitful analysis paradigm for studies of mammalian hibernation. Multiple studies have investigated the hibernation phenotype of 13-lined ground squirrels using high-throughput mass spectrometry. One study identified 232 differentially expressed proteins in skeletal muscle at various seasonal and hibernation time points (Anderson et al., 2016). Not surprisingly, the switch from carbohydrate to fatty acid metabolism during hibernation emerges from this data and demonstrated the time domain of this metabolic reorganization. Starting in the spring and leading up to entrance into torpor in the autumn, proteins involved in fatty acid metabolism rose higher and higher as the season progressed, whereas the opposite trend was seen with glucose metabolism proteins. This is consistent with well-known hyperphagia during this period to build up fat reserves in preparation for the hibernation season (Dark et al., 1989). The consistent increase in fatty acid metabolism proteins and decrease of glucose metabolism proteins continued at each time point until emergence of torpor in the spring, at which time a major switch occurred where fatty acid metabolism proteins significantly decreased and glucose metabolism proteins increased (Anderson et al., 2016). At the emergence of torpor, these animals feed for the first time since hibernation was initiated, and therefore must quickly reactivate carbohydrate metabolic pathways. When examined in this way, this untargeted proteomics data gives insight into the prolonged metabolic transition into a hibernation-ready state over the course of months and the quick transition out of it, a type of insight that may be missed with more traditional targeted studies of individual cellular processes.

Proteomics and phosphoproteomics have also been used to study BAT in ground squirrels (Vertommen et al., 2017) the tissue responsible for rapidly rewarming animals during arousal via non-shivering

thermogenesis (Nedergaard and Cannon, 1984). In BAT, the uncoupling protein UCP1 is expressed in mitochondria as a proton leak channel, allowing protons pumped out by the electron transport chain back into the mitochondrial matrix, without passing through complex V (ATP synthase) and releasing energy as heat instead. Liquid chromatography–mass spectrometry analysis of BAT in euthermic and hibernating ground squirrels indicated that most mitochondrial proteins did not change in protein level, suggesting changes in gene expression are not a major component of the metabolic warming process. However, phosphorylation levels of various proteins associated with BAT thermogenesis changed between euthermic and hibernating states, including hormone-sensitive lipase (HSL) and PDH. HSL cleaves tri- or diglycerides into fatty acids (Schweiger et al., 2006) and thus enzyme activation by phosphorylation may be an important step in mediating the supply of fuel for non-shivering thermogenesis during hibernation and arousal. Phosphorylation of PDH, as previously discussed, is a key player in the hibernation phenotype as it is the crucial point regulating the use of carbohydrate fuel by mitochondria, its inhibition contributing to the switch to lipid metabolism.

Enzymatic regulation of metabolism, particularly of liver glucose metabolism, has been a hallmark of research regarding the freeze tolerant wood frog. Post-translational modifications, particularly reversible protein phosphorylation, are primary regulatory factors in cryoprotectant glucose production when frogs begin to freeze (Cowan and Storey, 2001; Dieni et al., 2012; Dieni and Storey, 2010, 2011; Smolinski et al., 2017), however, only recently was a comprehensive evaluation of the wood frog phosphoproteome conducted (Hawkins et al., 2019). Freeze tolerance necessitates endurance of other stresses including cellular dehydration and anoxia, and hence an analysis of the liver phosphoproteome compared the responses to freezing with the separate responses to dehydration and anoxia alone. Phosphoproteins involved in glucose metabolism were abundant in the analysis (Fig. 3), which was not surprising given that glycogenolysis is highly active and whereas glycolysis and the pentose phosphate pathway are inhibited in order to funnel glucose-6-phosphate into glucose for export (Storey and Storey, 1984) (Churchill and Storey, 1994). High rates of glycogenolysis would normally be counteracted allosterically when glucose levels rose too high, but the massive amounts of glucose needed for cryoprotection require novel regulatory mechanisms for when this animal encounters stresses such as freezing and dehydration. While multiple glycogenolysis related enzymes were differentially phosphorylated, glycogen phosphorylase itself showed decreased phosphorylation at a non-canonical site (serine 430) but not at the serine 14 site that triggers phosphorylase *b* to *a* conversion, which may contribute to the unique pattern of glycogen phosphorylase regulation in this animal (Crerar et al., 1988). Furthermore, the synthesis of cryoprotectant glucose from glycogen would be inefficient if glycogenolytic end-products were able to flow through glycolysis and prior studies indicated that whereas hexose phosphate intermediates increased many-fold, fructose-1,6-bisphosphate and triose phosphate intermediates remained constant, suggesting a metabolic block at PFK1 to block carbon flow (Storey, 1987b). The results of this study give a potential mechanism for this metabolic block and showed increased phosphorylation of PFKFB2 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1). This bifunctional enzyme can both synthesize and degrade fructose-2,6-bisphosphate, a powerful activator of PFK. Phosphorylation of PFKFB2 promotes the phosphatase activity of the enzyme and lowers fructose-2,6-bisphosphate levels thereby reducing PFK activity (Fig. 3). Interestingly phosphorylation of PFKFB2 in dehydrated animals showed the opposite results even though dehydrating frogs also build up high glucose. Similarly, there was no statistical difference in PFKFB2 phospho-peptides in the anoxia-exposed animals, which is consistent with the need for anaerobic glycolysis to be active.

High-throughput tools such as those discussed here enable true discovery science. Capturing entire landscapes of biomolecules and how they are regulated to control adaptive responses reveals mechanisms that may otherwise go undetected through traditional targeted research approaches. The continued use of these technologies will drive new research directions and will surely produce insights that can be applied to problems we wish to solve.

5. Applications

The motivation to study organisms that are adapted to extreme environmental conditions comes in part from the desire to solve real world problems. Billions of years of evolution have produced natural answers to many problems that humans wish to solve. For example, heavier-than-air flight was not solved by humans de novo, but rather the pioneers of flight looked to nature for the design. Likewise, questions of a biological nature can be asked and are often answered by examples in nature.

5.1. Organ and tissue preservation

Our ability to supply organs and tissues has yet to meet the demand of those in need. The feasibility of preservation varies widely by organ and tissue, but the main limiting factor for successful transplantation of solid organs is time outside a living body (Giwa et al., 2017). While the focus of discussion is often on the imbalance between the donors available and the individuals in need, what is shocking is the high percentage of organs that are available for donation that do not maintain viability long enough for transplantation to occur. For example, more than 80% of available lungs and 70% of available hearts do not make it to a patient in need (Giwa et al., 2017). This is a multifactorial problem, but often the root cause is organ deterioration after removal from the donor which is exacerbated by the health of the organ pre-transplantation. Since organ viability is currently measured in hours, not only do patients need to find a donor within geographic proximity, but that donor must also be HLA compatible (Opelz et al., 1999). Improving organ preservation times from hours to days, week, or months, would not only dispel the geographic limitations and allow time for better donor-recipient matching, but would realize the goal of organ banking to buffer fluctuations between supply and demand as is the case with blood and plasma donation (Lewis et al., 2016).

The main sources of injury to organs outside of the body are oxygen and metabolic related stresses. These arise because in traditional organ harvesting/storage procedures, the organ is no longer perfused by the circulatory system to supply oxygen and drain metabolic wastes. The rate at which damage occurs is proportional to the metabolic rate of the tissue, therefore strategies to minimize damage have sought to reduce metabolic rate by lowering organ temperature. This has been most successful for tissues such as blood, plasma, semen and oocytes where cryopreservation is an option, but reliable cryopreservation of complex tissues and solid organs has yet to be realized (Taylor et al., 2019).

Three main strategies to improve organ preservation can be modeled using examples from nature: 1) whole-body freeze tolerance, 2) cold-temperature hibernation, and 3) warm-temperature hibernation. Freeze tolerant vertebrates such as the wood frog show that cryopreservation of complex tissues and organs is a reality. These animals regularly survive days at a time frozen at high sub-zero temperatures (Storey and Storey, 2017) and up to 6 months in Alaskan populations (Larson et al., 2014). During this time organ perfusion ceases since the blood is frozen and heart beat stops. To account for the lack of perfusion, organ metabolism is suppressed to a fraction of normothermic levels through a variety of mechanisms including transcriptional reprogramming, miRNA silencing, and posttranslational modifications to minimize ATP expenditure (Storey and Storey, 2017). The wood frog also avoids cryoinjury through the use of glucose as a cryoprotectant, and ice nucleating agents to control the size and location of ice crystals (Lee and Costanzo, 1998). Supercooling of body fluids can lead to snap freezing which is particularly damaging; however, the wood frog exists in a condition where ice is nucleated at high sub-zero temperatures

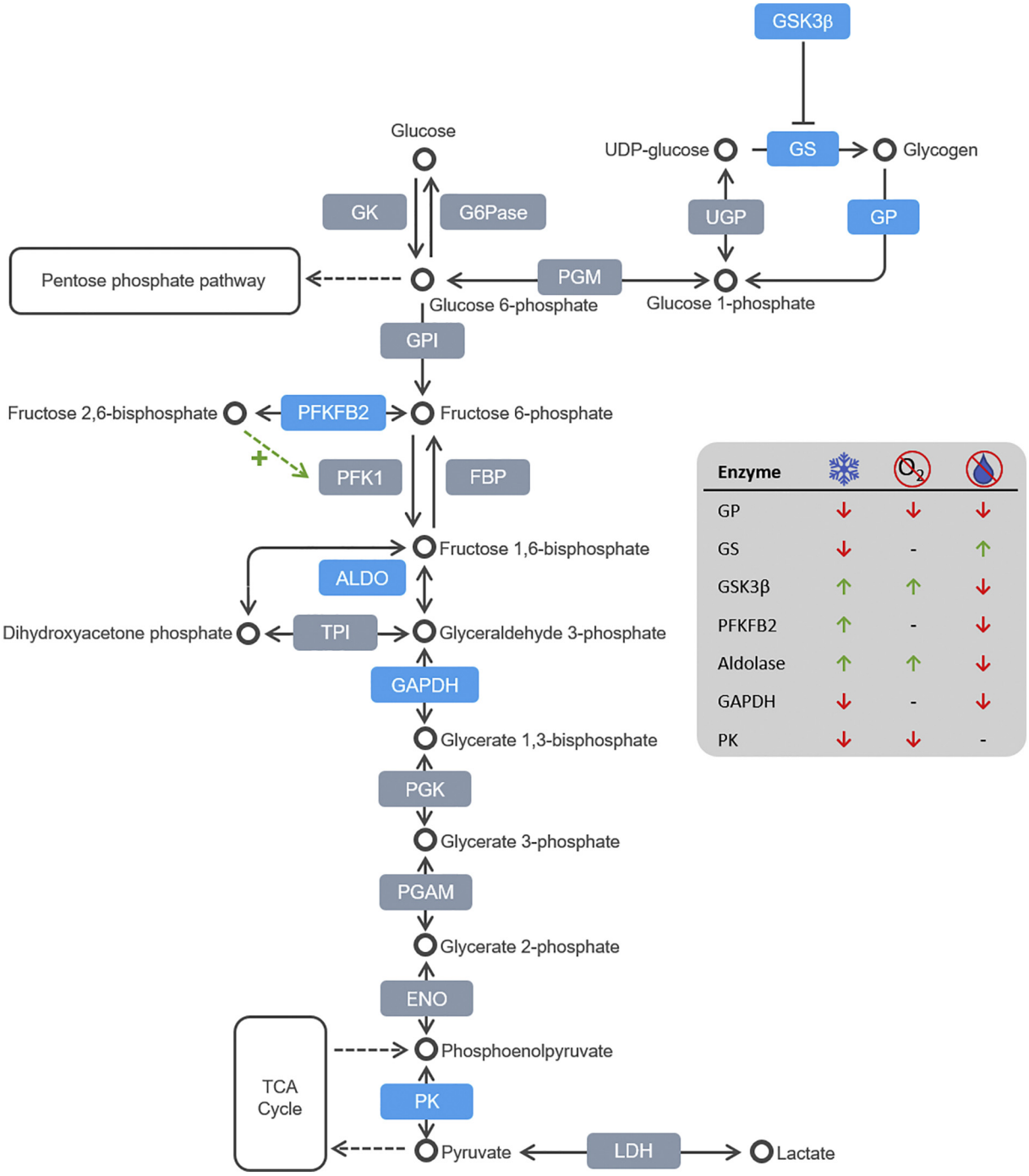

Fig. 3. Differential phosphorylation of enzymes of glucose metabolism in the liver of wood frogs under freezing, anoxia, or dehydration stress as determined by mass spectrometry phosphoproteome analysis. Enzymes in blue were found to be differentially phosphorylated in one or more experimental condition (24 h freezing, 24 h anoxia, or 40% dehydration). The direction of change in phosphorylation for each enzyme under each condition with respect to control animals is indicated in inset table. Adapted with permission from (Hawkins et al., 2019).

either by contact with ice crystals from the environment or by ice nucleating bacteria on the skin and in the gut (Lee and Costanzo, 1998). The wood frog displays no anticipatory cryoprotective responses, but rather waits until the onset of ice nucleation when the liver is rapidly activated to synthesize cryoprotective glucose that is then exported to all tissues and taken up to protect the cytoplasm from freezing (Storey and Storey, 1996). The fact that the wood frog enacts freeze tolerance adaptation within minutes rather than needing a prolonged acclimation period bodes well for application to organ preservation where often the availability of an organ from deceased donors is not foreseen. This means that implementing the correct cryopreservation protocols inspired by animals such as the wood frog can be done at the time and location of organ harvesting.

The latest innovation in organ transplantation is normothermic perfusion which attempts to place the harvested organ in an environment mimicking the human body, complete with a perfusate supplying oxygen and metabolic substrates (Hessheimer et al., 2019). The success of this strategy ultimately depends on how closely the perfusion environment matches the human body, and damage will result from misalignment between the two. Reducing metabolic rate of the organ is

analogous to decreasing time under perfusion. A lower metabolic rate demands less oxygen, produces less metabolic waste and therefore is a method to minimize damage in imperfect preservation environments. Mammalian hibernators, cold and warm, give multiple examples of how organ metabolism and perfusion rates can be minimized for extended periods of time without enduring damage. Cold hibernating mammals, those that allow their body temperature to drop to near ambient levels, show metabolic rate depression through temperature dependent (Geiser, 2004) and independent measures (Brown et al., 2012) coupled with reduced perfusion rates even in the brain (Frerichs et al., 1994). Animals such as the 13-lined ground squirrel appear to be resilient to damage associated with reduced perfusion and cold injury (Lindell et al., 2005). Determining and applying the mechanisms used by cold hibernators may be a stepping stone towards prolonged preservation until normothermic perfusion is perfected.

Using cold temperatures to aid metabolic rate depression comes with the added challenges of cold injury and cold ischemia-reperfusion damage. Many hibernating species have mastered this but humans have not. It is for this reason that the use of warm hibernation strategies is becoming an interesting avenue to pursue with regard to organ preservation (Hadj-Moussa and Storey, 2018b). Various mammalian species undergo hibernation/torpor where physiological and metabolic processes are reduced but without a dramatic reduction of body temperature. Notable are hibernating bears who show at least a 50% reduction in heart rate and a 75% reduction in metabolic rate during hibernation but their huge body mass (and insulating fur and fat) prevents body temperature from falling more than a few degrees (Evans et al., 2012; Tøien et al., 2011). However, this disconnect between metabolic rate and body temperature reduction shows the important feature that metabolic suppression is inducible in mammalian species without the aid of chilling. Elucidating the mechanisms that these animals use and applying them to current normothermic perfusion strategies could slow the rate at which damage accumulates and therefore extend the preservation window.

5.2. Therapeutics and treatments

5.2.1. Muscle atrophy

Biomedical applications of the adaptive strategies that deal with environmental challenges extend beyond tissue and organ preservation. During hibernation, large and small mammals remain dormant for many months at a time. The analogous situation for humans results in severe muscle disuse atrophy, including 30% or greater loss of muscle mass and strength (Bloomfield, 1997), yet mammalian hibernators show significantly less atrophy after similar periods of time (Lee et al., 2008; Lohuis et al., 2007a, 2007b). The anti-atrophy mechanisms involved have not been fully elucidated; however, regulation of proteolysis is a clear target. Hibernating black bears show reduced but equal rates of skeletal muscle protein synthesis and degradation (Lohuis et al., 2007a). Neural activation throughout hibernation may be one way that muscle mass is maintained even though load bearing is reduced. A study in 2012 added an interesting piece to the puzzle by showing that denervation of brown bear skeletal muscle led to the expected atrophy during the summer, but not when animals were hibernating (Lin et al., 2012). This means that a mechanism is in place during hibernation to regulate skeletal muscle mass independent of direct nervous control. Investigation into other signalling routes led to a study of serum from hibernating bears (Chanon et al., 2018). The authors showed that culturing human skeletal muscle cells in serum from hibernating bears increased muscle cell size and decreased protein degradation compared to serum from summer bears or fetal bovine serum. Identifying the factor(s) present in hibernating serum is the next step, but these results show promise for a muscle disuse atrophy therapeutic.

5.2.2. Diabetes and hyperglycemia

Diabetes is characterized by a prolonged elevation of blood glucose levels, which if left untreated lead to a variety of long-term complications including retinopathy, neuropathy, and cardiovascular disease (Nathan, 1993). In the short-term, hyperglycemia leads to oxidative stress, protein damage by non-enzymatic glycation, ketoacidosis and even death (Giacco and Brownlee, 2010; Singh et al., 2014). A blood glucose level of 10 mM in humans is considered high, and yet the wood frog regularly exhibits 200–300 mM blood glucose to protect against freezing damage with no apparent increase in overall protein glycation and an actual decrease in glycated hemoglobin (MacDonald et al., 2009). Interestingly, when whole blood was incubated with 400 mM glucose, hemoglobin glycation showed similar increases in wood frog and whole blood from rats suggesting an *in vivo* mechanism for protecting against hemoglobin glycation. Determining what this mechanism might be could help treat human diabetics with chronic hyperglycemia.

5.2.3. Ischemia and stroke

Ischemic stroke is characterized by a blockage or severe restriction of blood flow in areas of the brain causing localized and potentially cascading damage (Favate and Younger, 2016). Susceptibility and prognosis are influenced by multiple environmental, genetic, and epigenetic factors, but natural “stroke tolerant” models exist in nature. Multiple mammalian models approximate brain ischemia, notably hibernators show a significant reduction in brain perfusion without suffering ischemic damage (Frerichs et al., 1994). This has led to much interest in possible neuroprotective and biomedical interventions inspired by these animals (Dave et al., 2012; Drew et al., 2001; Zhou et al., 2001). While hibernation certainly holds important information about tolerating brain ischemia, they are not a perfect model for ischemic stroke. Unlike the sudden onset of ischemic stroke in humans, the onset of hibernation, particularly in obligate hibernators, is a gradual process where preparative mechanisms are active weeks to months in advance. A better model organism for studying rapid ischemia shock may be the freeze tolerant wood frog which exhibits no preparatory measures for freezing and there can be a point in time after freezing is initiated where the blood is frozen but the brain is not (Rubinsky et al., 1994). This certainly results in similar rapid ischemic conditions as those present during ischemic stroke, yet upon thawing the frog regains full motor capabilities and appears otherwise unscathed. The brain of the wood frog is understudied, and thus our knowledge of the neuroprotective mechanisms in this species is vastly outweighed by that of the physiology and metabolism of the liver, for example. It is known that while frozen and during thawing, whole brain levels of cell signalling cascades change (Greenway and Storey, 2000), some adjustment of metabolic activities take place (Cowan and Storey, 2001), and antioxidant defenses may be active (Joanisse and Storey, 1996), however, the crucial period between blood freezing and brain freezing has yet to be examined. Analysis of this period may indicate the activation of neuroprotective processes, or potentially intrinsic ischemia tolerance which will increase our understanding of how to mitigate damage by ischemia and ischemic stroke.

6. Conclusion

The case for studying adaptations employed by animals that survive extreme environment stress extends beyond a purely interest-led pursuit. Rather, nature is a hotbed of inspiration for solving many of our outstanding technological and biomedical problems. The optimization algorithm that is evolution has supplied nature’s answers to these problems and the new advances and directions discussed here illustrate the ever more nuanced understanding that we have of these systems. While there is undoubtedly much to learn about the adaptations that animals employ to survive, new techniques, analytical technologies, and research paradigms will drive these areas of study and will allow us to capture and harness these answers for our benefit.

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Declaration of competing of interest

The authors report no conflicts of interest

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