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Review

# Tribute to P. L. Lutz: putting life on 'pause' – molecular regulation of hypometabolism

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**Summary**

**Entry into a hypometabolic state is an important survival strategy for many organisms when challenged by environmental stress, including low oxygen, cold temperatures and lack of food or water. The molecular mechanisms that regulate transitions to and from hypometabolic states, and stabilize long-term viability during dormancy, are proving to be highly conserved across phylogenic lines. A number of these mechanisms were identified and explored using anoxia-tolerant turtles as the model system, particularly from the research contributions made by Dr Peter L. Lutz in his explorations of the mechanisms of neuronal suppression in anoxic brain. Here we review some recent advances in understanding the biochemical mechanisms of metabolic arrest with a focus on ideas such as the strategies used to reorganize metabolic priorities for ATP expenditure, molecular controls that suppress cell functions (e.g. ion pumping, transcription, translation, cell cycle arrest), changes in gene expression that support hypometabolism, and enhancement of defense mechanisms (e.g. antioxidants, chaperone proteins, protease inhibitors) that stabilize macromolecules and promote long-term viability in the hypometabolic state.**

Key words: metabolic rate depression, anoxia tolerance, hibernation, reversible protein phosphorylation, signal transduction, stress-induced gene expression, cell cycle arrest, antioxidant defense.

## Introduction

"*Turn off your mind, relax and float downstream, it is not dying, it is not dying*" from 'Tomorrow Never Knows' by Lennon and McCartney, 1966

Metabolic rate depression is a widespread survival strategy found across all kingdoms of life. When challenged by environmental or physiological constraints that make normal life impossible and challenge viability, many organisms retreat into a hypometabolic, or even ametabolic, state. Sometimes this involves a change to another life stage (e.g. production of seeds, spores, eggs, cysts, etc.) that can 'wait out' the stress, but often multicellular organisms can make reversible excursions into hypometabolic states that can last for days, months or sometimes years. In animals, hypometabolic states are integral parts of phenomena including hibernation, estivation, diapause and anaerobiosis, to name a few, and virtual ametabolic states occur in anhydrobiosis and some forms of extreme cold tolerance (for reviews, see Hochachka and Guppy, 1987; Clegg, 2001; Denlinger, 2002; Storey, 2002; Storey and Storey, 1990; Storey and Storey, 2004; Hochachka and Lutz, 2001; Heldmaier et al., 2004). Hypometabolism can be a seasonal phenomenon (e.g. winter hibernation in small mammals) or linked with a particular life stage (e.g. an obligate diapause at one stage of insect development) so that circannual or developmental considerations are often involved. In other cases, entry into a hypometabolic state is opportunistic, occurring repeatedly over a lifetime whenever environmental conditions are poor (e.g. estivation in response to water/food deprivation; anaerobiosis in response to lack of oxygen). Furthermore, in some systems, the production of dormant forms (e.g. eggs, embryos, spores, seeds) that refrain from hatching despite developmental preparedness and favorable environmental conditions may have a different purpose and provide 'bet-hedging' against future catastrophes by instituting variable emergence timing (Evans and Dennehy, 2005). All of these examples of hypometabolism have been well studied as independent phenomena. However, it is now well recognized that they share common biochemical mechanisms and my laboratory has been particularly interested in the commonalities of metabolic regulation that underlie transitions to/from hypometabolic states (for reviews, see Storey and Storey, 1990; Storey and Storey, 2004).

## Anoxia tolerance and the 'terrrtle' model

For the researcher, hypometabolism induced by oxygen depletion is one of the easiest models to manipulate and study experimentally. Excellent vertebrate (e.g. turtles, carp) and invertebrate (e.g. various mollusks, some insects) models of hypoxia and/or anoxia tolerance have been studied extensively (e.g. De Zwaan and Putzer, 1985; Brooks and Storey, 1997; Lutz and Nilsson, 2004; Haddad, 2006). Anoxia-induced metabolic suppression is typically profound; for example, anaerobic metabolic rate is often <5% in intertidal mollusks and 10–20% in turtles of the corresponding aerobic rate at the same temperature (De Zwaan and Putzer, 1985; Storey and Storey, 1990; Jackson, 2000). Such strong metabolic suppression allows a maximal extension of the time that endogenous carbohydrate reserves can support life and compensates, at least in part, for the much lower yield of ATP from the anaerobic fermentation of carbohydrate *via* glycolysis compared with the yield from oxygen-based mitochondrial oxidation of fuels (Hochachka and Somero, 2002).

Our laboratory has a long-standing interest in the biochemical mechanisms of anoxia tolerance in mollusks, freshwater turtles, and several other models, ranging from studies of intermediary metabolism and enzyme regulation, through analysis of intracellular signal transduction mechanisms, evaluation of the role of antioxidant defenses and, most recently, studies of anoxia-induced gene expression (for reviews, see Storey, 1996; Storey, 2004a; Storey, 2007; Brooks and Storey, 1997; Hermes-Lima et al., 2001; Larade and Storey, 2002). Our work with turtles has examined not only submergence hypoxia/anoxia in adult freshwater turtles but also freeze tolerance and its attendant anoxia/hypoxia stress in hatchling painted turtles (reviewed in Storey, 1996; Storey, 2004a; Storey, 2006a; Storey, 2007). Our interests in the mechanisms of turtle anoxia tolerance led to frequent contact with the laboratory of Dr Peter L. Lutz, and over the years, one of us (K.S.) traveled several times to Florida (not surprisingly, during the Canadian winter) to interact with Peter and his 'terrrtles', as his Scottish brogue pronounced them. Peter's interests in respiratory and neurophysiology of turtle anoxia tolerance dovetailed with our lab's interests in metabolic biochemistry, resulting in a 1997 joint review on the respiratory, neurological and biochemical adaptations of animals to low oxygen (Lutz and Storey, 1997). Research in both our labs led to realization of the central role that metabolic rate depression plays in anoxia survival. Peter's work focused on ion channel arrest and the role of adenosine as the neurotransmitter mediating the suppression of neuronal activity in the anoxic brain (for reviews, see Lutz and Nilsson, 1997; Lutz and Nilsson, 2004), whereas our lab studied the control of intermediary metabolism during anoxia, particularly the role that reversible phosphorylation, catalyzed by protein kinases and protein phosphatases, has in the suppression of enzyme/protein function under anoxic conditions (for reviews, see Storey, 1996; Storey, 2004a; Storey, 2004b; Storey and Storey, 1990; Storey and Storey, 2004). Most recently, both our labs began to explore the role of differential gene expression in turtle anoxia survival (Cai and Storey, 1996; Willmore et al., 2001a; Prentice et al., 2003; Prentice et al., 2004; Milton et al., 2006; Storey, 2004a; Storey, 2006b). Recent review articles from our laboratory have elaborated on the important contributions to understanding natural anoxia tolerance made by Peter Lutz (Storey, 2007) and by another Peter – the 'godfather' of comparative biochemistry, Peter W. Hochachka (Storey, 2004b). Those and other recent articles (Storey, 2004a; Storey and Storey, 2004) examined the development of ideas in the field and showed how an initial focus on the regulation of anaerobic energy metabolism provided the framework that has since allowed other labs to explore the regulation of many other areas of metabolic function in anoxia.

## Principles of metabolic rate depression

In the present article, we examine the conserved principles of metabolic rate depression across the animal kingdom, integrating recent advances not only in anoxia tolerance but also in other hypometabolic states including hibernation and estivation. In particular, we will focus on new horizons in research on hypometabolism. What are the global events in cell/organism metabolism that need attention in order to power down and enter a dormant state? What are the mechanisms by which these events are controlled and coordinated?

In general, we can identify several criteria as necessary for long-term survival in a hypometabolic state. These are: (1) global metabolic rate suppression, (2) fuels for long-term survival, including adequate and appropriate supplies, altered patterns of fuel use, and mechanisms to limit internal pollution by accumulated end products, (3) triggering and signal transduction mechanisms to deliver and coordinate metabolic responses by all cells and organs, (4) reorganization of metabolic priorities for ATP expenditure both within cells and between organs, including differential regulation of many ATP expensive processes such as ion pumping, transcription, translation, growth and development, (5) changes in gene expression, and (6) enhancement of defense mechanisms that stabilize macromolecules and promote long-term viability in the hypometabolic state. Each of these criteria is considered below. Some receive only brief attention because they have been well reviewed elsewhere. Others are discussed in greater detail, in particular to highlight new areas of interest and very recent advances.

## Global metabolic rate suppression

Hypometabolic systems are characterized by a strong net suppression of organismal metabolic rate, targeting and coordinating both ATP-utilizing and ATP-generating cell functions. Net suppression is typically >80%, often 95% or greater, and nearly 100% in cryptobiotic systems. In some cases, hypometabolism is facilitated by reduced body temperatures such as during winter diapause or quiescence in invertebrates, or in mammalian hibernation when metabolic arrest (combined with suppression of the hypothalamic set-point for body temperature control) results in a drop in body temperature to near ambient (Storey and Storey, 1990; Denlinger, 2002; Geiser,

2004; Heldmaier et al., 2004). Aquatic organisms in hypoxic water also seek out lower water temperatures to minimize metabolic rate (Tattersall and Boutilier, 1997). Several factors can contribute to global metabolic suppression, including reduced physiological activities (e.g. animals move less and often do not eat while in hypometabolic states), changes in extrinsic factors (e.g. partial pressures of $O_2$ and $CO_2$, pH and temperature), and changes in intrinsic factors (e.g. specific biochemical inhibition of metabolic activities). Several studies have indicated that extrinsic parameters can account for a significant portion of overall metabolic inhibition (Barnhart and McMahon, 1988; Guppy et al., 2000; Langenbuch et al., 2006; Michaelidis et al., 2007).

## Fuel supplies, energy metabolism and handling of end products

Fuel supplies and the control of energy metabolism were among the very first topics addressed in studies of hypometabolic states and have been reviewed many times, including in the references cited below. Seasonal or life-stage-specific dormancies include accumulation of adequate 'on-board' metabolic fuel reserves that are laid down before dormancy begins. For aerobic states this typically involves accumulation of large triglyceride pools; for example, late-summer hyperphagia ensures that mammalian hibernators build up huge white adipose depots that increase body mass by ~50% (Wang and Lee, 1996). By contrast, anoxia-tolerant species amass large glycogen reserves to fuel anaerobic glycolysis (Hochachka and Somero, 2002). Most cold-hardy and anhydrobiotic organisms do the same, but a high proportion of their glycogen is directed into the synthesis of protective polyols and sugars (Storey, 1997a; Clegg, 2001).

Necessary controls are also implemented to direct the appropriate use of fuels during dormancy. For example, mammalian hibernators upregulate a variety of genes whose protein products support lipid catabolism during torpor (e.g. fatty acid binding proteins, lipoprotein lipase, subunits of electron transport chain complexes) (Carey et al., 2003; Storey and Storey, 2004). Anoxia-tolerant marine invertebrates use reversible protein phosphorylation controls on key enzymes of glycolysis to achieve both net glycolytic suppression and a re-routing of carbohydrate flow into alternative end products that are associated with enhanced ATP production (Storey and Storey, 1990).

Hypometabolic states also typically require novel solutions to minimize internal 'pollution' from the accumulation of metabolic end products over the long term; this is most prominent in anoxic systems. Among the solutions used are (a) a switch to the production of volatile or diffusible end products (e.g. propionate, acetate, ethanol) that can be excreted, (b) high buffering capacities to minimize metabolic acidosis resulting from the build-up of anaerobic end products, (c) novel solutions for long-term storage of end products (e.g. anoxic turtles store lactate in their shells), or (d) putting end products to useful purposes (e.g. urea accumulation as a result of protein catabolism aids desiccation resistance in estivation) (Hochachka and Mommsen, 1983; Hochachka and Somero, 2002; Storey, 2002; Jackson, 2004).

## Triggering and signal transduction

Mechanisms are needed to detect, relay and coordinate responses both within and between cells and organs in order to make transitions to and from hypometabolic states. Knowledge in this field is still sketchy, but a few new developments are noted below. Hypometabolic states are typically triggered as a response to a stress that threatens normal life. In some cases, primary stress detection can occur at the level of each individual cell (e.g. low oxygen), whereas in other cases coordination by the central nervous system is required, especially when entry into hypometabolism is 'hard-wired' (e.g. in mammalian hibernation or many forms of diapause). For example, in the silkmoth, *Bombyx mori* L., the production of maternal diapause hormone acts to arrest embryogenesis, resulting in the production of diapause eggs (Denlinger, 2002). Similarly, a hibernation induction trigger (HIT) has long been proposed for hibernating mammals and available evidence tends to indicate that it is some type of opiate since a synthetic alpha opioid, D-Ala2-D-Leu5-enkephalin, mimics HIT effects (Horton et al., 1998; Borlongan et al., 2004). New work (Blackstone et al., 2005) has also created great excitement in the field of metabolic arrest by demonstrating that hydrogen sulfide is a very powerful inducer of metabolic depression. Mice exposed to 80 p.p.m. $H_2S$ showed a 50% drop in oxygen consumption within 5 min, and after 6 h of exposure, metabolic rate was stable at just 10% of normal and core body temperature had fallen to ambient. These effects were fully reversible when $H_2S$ was removed. An explosion of research on $H_2S$ effects is underway. Indeed, brand new work has linked the cardioprotective effects of ischemic preconditioning to the actions of endogenous $H_2S$ (e.g. Pan et al., 2006; Johansen et al., 2006; Bian et al., 2006) and new signalling roles for $H_2S$ have been identified in gastrointestinal tract and liver (Fiorucci et al., 2006). This will be a very exciting area of research over the next few years. These findings are also intriguing because they add to the list of simple, small, primordial molecules that are now recognized as having biological signalling actions (e.g. nitric oxide, carbon monoxide, hydrogen peroxide) (Rhee et al., 2005).

Triggering and signal transduction in anoxia-tolerant systems has also received considerable attention. Here we need to differentiate those signals that are designed to improve/rescue oxygen-based metabolism under oxygen-limited conditions from those signals that are designed to induce metabolic arrest and protect tissues from injury over long-term anoxia. Much recent work has focused on the former, and particularly on the role of the hypoxia inducible transcription factor 1 (HIF-1) in mediating gene expression responses in hypoxia. Under low oxygen conditions, the alpha subunit HIF-1 is stabilized, allowing it to migrate to the nucleus, bind with the beta subunit, and trigger the upregulation

of genes containing a hypoxia response element (Semenza, 2003). Genes under HIF-1 control typically produce proteins that serve one of two purposes: (a) to enhance oxygen delivery to cells by stimulating capillary growth or increasing numbers of red blood cells, or (b) to enhance the capacity for glycolytic ATP production. HIF-1 activation also seems to occur in response to anoxia exposure in some anoxia-tolerant species but the question is why. Excellent facultative anaerobes (such as turtles) make transitions into anoxia without showing 'traditional' actions of HIF-1 such as upregulation of glycolytic enzyme activities (Willmore et al., 2001b). Angiogenic and erythropoietic actions of HIF-1 are also of no value if oxygen levels are zero. Hence, a question of current interest to us is whether the multiple categories of HIF-1 effects are differentially regulated to achieve altered goals in anoxia-induced hypometabolism. For example, HIF-1 action in growth arrest may be a key action needed in anoxic systems whereas angiogenic action may be inhibited. Some emerging ideas about this are discussed in later sections.

Other signalling mechanisms involved in anoxia tolerance derive from products of ATP degradation, providing a link between metabolic arrest and energy restriction. Hypoxia or anoxia stress produces a characteristic fall in cellular ATP levels (although typically just transient in hypoxia/anoxia tolerant species) and leads to the production of ATP degradation products that have metabolic effects. These include: (a) AMP that can activate catabolic and inhibit anabolic pathways both by allosteric effects on enzymes and by activating the AMP-dependent protein kinase (Hardie and Sakamoto, 2006), (b) IMP + $NH_4^+$ that are produced by AMP deaminase in tissues where stabilization of adenylate energy charge is key (e.g. working muscle); both have allosteric effects on enzymes (Mommsen and Hochachka, 1988), and (c) adenosine and inosine that are produced from AMP and IMP by specific 5′ nucleotidases. Adenosine is well known to have a key role as the neurotransmitter mediating the suppression of neuronal activity in anoxia-tolerant species (Lutz and Nilsson, 2004) including ion channel arrest (Buck, 2004; Buck and Pamenter, 2006), whereas new research suggests an equally critical action of inosine in triggering antioxidant defense responses in response to hypoxia, hyperoxia or $H_2O_2$ insult (Gelain et al., 2004; Buckley et al., 2005; Tomaselli et al., 2005). This latter is very exciting for two reasons: (a) the enzyme that makes inosine, 5′ nucleotidase cytosolic II isozyme (NT5C2), is upregulated in brain of adult turtles *Trachemys scripta elegans* in response to anoxia (Storey, 2007), and (b) enhancement of antioxidant defenses is seen widely in hypometabolic states, as discussed later in this article.

## Reorganization of metabolic priorities

Entry into a hypometabolic state involves differential regulation of a vast array of metabolic functions both within cells and between organs, ranging from a complete shut off of many nonessential processes to partial, or perhaps even full, maintenance of processes that are critical to viability. Studies with turtle hepatocytes illustrated this well. Under anoxia, cells showed a net 94% decrease in ATP turnover but with very different changes in the proportion of ATP use by five main ATP-consuming processes (Hochachka et al., 1996). As a result, the $Na^+$,$K^+$-ATPase became the dominant user of ATP in anoxic hepatocytes, consuming 62% of total ATP turnover compared with 28% in normoxia, whereas protein synthesis and protein degradation were suppressed by >90% and gluconeogenesis and urea synthesis were virtually halted. A similar strong suppression of protein synthesis was reported by Lutz and collaborators when monitoring radiolabeled amino acid incorporation into organ protein pools in turtles *in vivo* (Fraser et al., 2001). Hibernating ground squirrels also show suppression of protein synthesis during torpor but to differing degrees in different organs (Frerichs et al., 1998; Hittel and Storey, 2002); for example, the protein synthesis machinery in the key thermogenic organ, brown adipose tissue (BAT), appears to stay fully functional during torpor but is strongly suppressed in brain and kidney. Use of $^3$H-uridine and $^{14}$C-leucine labelling showed that both transcription and translation rates were strongly reduced in organs of hibernating hamsters (Osborne et al., 2004).

## Reversible protein phosphorylation – main mechanism for metabolic reorganization

Reversible protein phosphorylation (RPP) is the most widespread and powerful mechanism available to cells for making fast, stable changes to the activity state of cellular proteins and, therefore, it is not surprising that it plays a dominant role in regulating hypometabolism (Storey and Storey, 2004). RPP regulates the activities and kinetic properties of huge numbers of metabolic enzymes, particularly those with gate-keeping or rate-limiting roles, and frequently also alters their binding interactions with other proteins and their subcellular locations. Many kinds of functional proteins including membrane receptors and transporters, ion channels and ion motive ATPases are also regulated by RPP, as are many proteins involved in gene transcription, protein synthesis, protein degradation and cell cycle regulation. Signal transduction cascades also exploit protein phosphorylation events for transmission and amplification of signals starting from membrane receptors, spreading through multiple intermediary steps and ending in the alteration of protein function or gene expression (Cowan and Storey, 2003; MacDonald, 2004).

The earliest demonstration of the role of RPP in hypometabolism came from studies of marine mollusks, where anoxia-induced phosphorylation-mediated inactivation of pyruvate kinase (PK) was shown to regulate the fate of glycolytic carbon between aerobic and anoxic routes of catabolism (reviewed by Storey and Storey, 1990). Later, RPP was also found to coordinate phosphofructokinase and glycogen phosphorylase in anoxia, developing the idea of overall glycolytic pathway suppression by RPP. The link to the control of hypometabolism in general came with the realization

that the same mechanism also applied to glycolytic suppression in situations of aerobic metabolic arrest including estivation and hibernation and, furthermore, that in aerobic systems, RPP controls extended to the suppression of enzymes in mitochondrial oxidative catabolism such as pyruvate dehydrogenase (Storey, 1997b; Brooks and Storey, 1997; Storey and Storey, 1990). Given these RPP controls on enzymes of ATP-producing catabolic pathways in hypometabolic systems, the search was then extended to show that RPP also coordinated the consumption of ATP by functional proteins (e.g. transmembrane carriers, chaperones, etc.) and anabolic pathways (e.g. lipid and protein biosynthesis) (Hochachka and Lutz, 2001; Bickler et al., 2001; Storey and Storey, 2004). Below we discuss some recent studies that have further extended the known metabolic functions that are regulated and coordinated by RPP in hypometabolic states.

### *Membrane ion channels and ion motive ATPases*

Maintenance of membrane potential difference is critical for cell viability, and in multiple situations of hypometabolism it is clear that transmembrane sodium and potassium gradients are maintained but at much reduced rates of ATP turnover; studies with both anoxic turtles and estivating frogs support this (Buck and Hochachka, 1993; Flanigan et al., 1993). This is achieved *via* suppression of ion movements across membranes in both directions, both facilitated flow through ion channels and active, ATP-driven ion pumping. The need for this can be appreciated when it is realized that the $Na^+$,$K^+$-ATPase alone may utilize 5–40% of total ATP turnover in different cell types (Clausen, 1986). The concept of 'channel arrest' was put forward by Hochachka (Hochachka, 1986) and considerable evidence for this mechanism has accumulated in studies with turtle brain as well as for the concept of 'spike arrest' (a strong decrease in neuronal excitability under anoxia), much of it arising from research done by Lutz and collaborators (Hylland et al., 1997; Perez-Pinzon et al., 1992; Bickler et al., 2001; Hochachka and Lutz, 2001; Lutz and Nilsson, 2004). Although other regulatory mechanisms are also involved, RPP controls have been widely identified in the process, affecting voltage-gated ion channels ($Na^+$, $Ca^{2+}$, $K^+$) and membrane receptors (e.g. *N*-methyl-D-aspartate-type glutamate receptor) in anoxia-tolerant turtles (Hochachka and Lutz, 2001; Bickler et al., 2001; Bickler and Buck, 2007).

Suppression of $Na^+K^+$-ATPase activity by RPP was specifically demonstrated in hibernating mammals (MacDonald and Storey, 1999) and multiple proteins of $Ca^{2+}$ metabolism are also regulated during hibernation (Malysheva et al., 2001). We recently showed that RPP also regulates $Na^+$,$K^+$-ATPase during estivation in foot muscle and hepatopancreas of the land snail *Otala lactea* (Ramnanan and Storey, 2006a), which indicates a broad phylogenetic conservation of the mechanism. Snail $Na^+$,$K^+$-ATPase showed distinctly different properties in the estivating *versus* active state: maximal activity fell by about one-third, affinity for Mg.ATP was reduced ($K_m$ was 40% higher), and activation energy (derived from Arrhenius plots) was increased by ~45%. Foot muscle $Na^+$,$K^+$-ATPase from estivated snails also showed reduced affinity for $Na^+$ substrate and $Mg^{2+}$ activator ($K_m$ $Na^+$ rose by 80% increase, $K_a$ $Mg^{2+}$ increased by 60%). Immunoblotting revealed no change in total enzyme protein during estivation, but *in vitro* incubations that manipulated the activities of endogenous kinases and phosphatases found a major change in phosphorylation state. $Na^+$,$K^+$-ATPase from estivating snails proved to be a high-phosphate, low-activity form, whereas dephosphorylation returned the enzyme to the high-activity state characteristic of active snails. Stimulation with protein kinases A, C or G mimicked the changes in enzyme properties that were seen during estivation, whereas treatments with protein phosphatase 1 or 2A had the opposite effect. Phosphorylation of the catalytic alpha-subunit of $Na^+$,$K^+$-ATPase is well known in mammalian systems (Ewart and Klip, 1995; Bertorello and Katz, 1995), as well as the action of PKA or PKC altering substrate affinity in response to various signals (Bertorello et al., 1991; Beguin et al., 1994). Cyclic GMP-dependent protein kinase (PKG) has been implicated in regulating enzymatic responses to anoxia in several marine molluscs (Brooks and Storey, 1990; Michaelidis and Storey, 1990; Larade and Storey, 2002) and was singled out as the kinase mediating estivation-induced phosphorylation of PK in *O. lactea* (Brooks and Storey, 1994). This strongly suggests that PKG may also be the physiological regulator of $Na^+$,$K^+$-ATPase during snail estivation, indicating a major physiological role for this kinase in hypometabolism across the Mollusca and perhaps even in other phyla.

### *Glucose-6-phosphate dehydrogenase and the pentose phosphate pathway*

To date, studies of the regulation of enzymes during hypometabolism have largely focused on central catabolic pathways, such as glycolysis and lipolysis, but clearly other pathways must also be regulated. One candidate is the pentose phosphate pathway (PPP), also known as the hexose monophosphate shunt. The PPP has multiple functions in cells. It is the primary source of NADPH reducing power for most biosynthetic reactions (e.g. fatty acid synthesis) and for the production of reduced forms of antioxidants (e.g. glutathione, thioredoxin). Carbon shuffling within the PPP also produces the ribose needed for DNA and RNA synthesis as well as 3–7 carbon sugars or sugar phosphates for many uses. Carbon entry into the PPP is gated by glucose-6-phosphate dehydrogenase (G6PDH), the first of two NADPH-generating reactions in the pathway. G6PDH is often considered to be a 'housekeeping' enzyme but recent work has shown that modulation of G6PDH activities in various tissues (particularly liver) has an important impact on cell growth, nutrient processing, antioxidant defense and death (Kletzien et al., 1994; Tian et al., 1998). In a new study of hepatopancreas G6PDH from the land snail, *O. lactea,* we reported the first evidence of RPP-mediated changes in the properties of G6PDH between active and hypometabolic (estivating) states (Ramnanan and Storey, 2006b). During estivation G6PDH activity increased by 50%, substrate affinity improved ($K_m$ G6P decreased by 50%), and sensitivity to citrate

activation increased ($K_a$ magnesium citrate decreased by 35%). These changes were linked with a change in the abundance of low- *versus* high-phosphate forms of the enzyme; the low-phosphate form of G6PDH dominated in active snails (57% of total activity) whereas the high-phosphate form dominated during estivation (71%). The high-phosphate form also showed reduced sensitivity to urea inhibition and greater resistance to thermolysin proteolysis, indicating greater structural stability of the enzyme during dormancy. The interconversion of G6PDH between active and estivating forms was linked to the actions of PKG and protein phosphatase 1 and, in contrast with the effects of RPP on glycolytic enzymes in *O. lactea* (Brooks and Storey, 1997), the phosphorylation-mediated changes in G6PDH argued for a more active and more stable form of the enzyme during dormancy.

We proposed that the prominent function of G6PDH in antioxidant defense was the reason for the estivation-responsive modification of the enzyme. As discussed in a later section, improved antioxidant defenses are a common theme across all forms of hypometabolism and since the backbone of antioxidant defense is NADPH reducing power, the key role of G6PDH in gating the PPP can be appreciated. Indeed, G6PDH activity is elevated in response to oxidative stress in systems ranging from yeast to humans (Ursini et al., 1997), whereas inhibited and/or reduced G6PDH activity has been correlated with reduced antioxidant defense capacities, ROS-related cellular damage and ROS-induced cell death (Tian et al., 1999).

## Hypometabolism and transcriptional suppression – recent advances

Suppression of protein translation has been widely documented in hypometabolic systems, both *via* measured reductions in overall protein synthesis rates and *via* the use of western blotting with phospho-specific antibodies to show major changes in the proportions of the phosphorylated forms of key ribosomal proteins regulating initiation (e.g. eIF2α, 4E-BP1) or elongation (e.g. eEF2) (Storey and Storey, 2004; van Breukelen et al., 2004; Horman et al., 2005). Not surprisingly, then, overall rates of gene transcription are also suppressed. It is estimated that 1–10% of cellular energy is devoted to transcription, depending on the tissue (Rolfe and Brown, 1997) and this significant metabolic cost must be addressed when organisms enter hypometabolic states. Reductions in the rate of global gene transcription of 65–90% have been seen as responses to anoxia in brine shrimp (*Artemia franciscana*) and intertidal snails (*Littorina littorea*) (van Breukelen et al., 2000; Larade and Storey, 2002) and during hibernation in mammals (Bocharova et al., 1992; van Breukelen and Martin, 2002; Osborne et al., 2004).

A variety of mechanisms for transcriptional suppression and/or gene silencing have been uncovered in recent years. Included are mechanisms that focus at the level of gene transcription such as histone modifications or the regulation of RNA polymerase II activity, as well as post-transcriptional gene silencing mechanisms such as the inhibitory actions of short (micro) RNAs. The latter are small non-coding RNA transcripts (18–25 nucleotides in length) that are known to regulate gene expression by binding to target mRNAs to either inhibit their translation or direct them into degradation pathways (Bartel, 2004; Farh et al., 2005). Recent studies, especially in plants, are showing that short RNAs play a role in environmental adaptation (Dalmay, 2006) and another study (Dresios et al., 2005) has implicated microRNAs in the regulation of global protein synthesis under normal (37°C) *versus* cold-stress (32°C) conditions in cultured mouse neuroblastoma cells. Although this mouse study does not approach the level of cold stress that many organisms must deal with, it does provide the intriguing suggestion that microRNAs could have a role to play in cellular responses to cold and/or other environmental stresses. It will be exciting to see how this novel mechanism is applied in the regulation of hypometabolism.

As an initial foray into the investigation of mechanisms of transcriptional suppression during hypometabolism, recent studies in our laboratory have evaluated selected mechanisms of global transcriptional control in hibernating mammals. Three mechanisms were examined (Table 1) and these and others deserve to be explored in multiple systems of hypometabolism in order to elucidate the pattern(s) of transcriptional suppression in dormant states.

### *Histone modification*

Covalent modifications of histones are a crucial component of epigenetic events that regulate chromatin structure and gene function (Hassan and Zempleni, 2006). Multiple modifications of histones are known including phosphorylation, acetylation, methylation, biotinylation, ubiquitylation, sumoylation and

Table 1. *Effect of hibernation on indices of transcription and cell cycle regulation in skeletal muscle of thirteen-lined ground squirrels*

| | Ratio hibernating:euthermic value |
|---|---|
| Histone deacetylase | |
| Activity | 1.82±0.20 |
| HDAC1 protein | 1.21±0.05 |
| HDAC4 protein | 1.48±0.09 |
| Histone 3 | |
| Total H3 protein | 0.99±0.16 |
| Phospho-H3 | 0.61±0.09 |
| Acetyl-H3 | 0.75±0.04 |
| RNA polymerase II | |
| Activity | 0.57±0.04 |
| Total Pol II protein | 1.05±0.19 |
| Phospho-Pol II | 1.79±0.21 |

Values are means ± s.e.m.; $N$=3–4 independent determinations on preparations from different individuals. Hibernating and euthermic values are significantly different ($P$<0.05).

Data are modified from Morin and Storey (Morin and Storey, 2006).

ADP-ribosylation (Holbert and Marmorstein, 2005; Hassan and Zempleni, 2006). Histone acetylation makes chromatin more accessible to the transcriptional machinery and is known to be a prominent mechanism of transcriptional activation (Hebbes et al., 1988). Phosphorylation of histone H3 at serine residue 10 has also been linked with transcriptional activation (Cheung et al., 2000). Using western blotting we evaluated histone H3 responses during hibernation. Total histone H3 content remained constant between euthermic and hibernating states but the amounts of both phosphorylated histone H3 (Ser 10) and acetylated histone H3 (Lys 23) were reduced by 38–39% during hibernation in skeletal muscle of 13-lined ground squirrels *Spermophilus tridecemlineatus*, as compared with euthermia (Morin and Storey, 2006). Both of these modifications of histone H3 are consistent with an overall decrease in transcriptional activity during hibernation. Histones H2A/H2B and H4 are also subject to acetylation that regulates their activity and these may also be modified in a parallel way during hibernation. Interestingly, studies of the response to anoxia by the nematode, *Caenorhabditis elegans*, produced the same result. Levels of the phosphorylated forms of cell-cycle-regulated proteins were virtually undetectable in embryos exposed to anoxia but reappeared during aerobic recovery (Padilla et al., 2002). These included histone H3 as well as a variety of proteins that are prominently phosphorylated during mitosis and recognized by the MPM-2 antibody. Hence, phosphorylation regulation of histone H3 may prove to be a widely conserved component of metabolic arrest across phylogeny (from nematodes to mammals) operating in situations of both aerobic and anoxic hypometabolism.

### *Control of histone deacetylases*

The acetylation state of histones is controlled by acetylase and deacetylase enzymes and regulation of these is key to histone modification in response to stimuli (Holbert and Marmorstein, 2005). Given the above results for histone H3 in hibernators, we predicted that histone deacetylase (HDAC) activity or protein content would be modified during hibernation to promote deacetylation. At least 18 isoforms of HDAC are known in mammals, belonging to three different classes (Lin et al., 2006). We measured both total HDAC activity and the relative protein levels of two prominent HDACs, HDAC1 and HDAC4 belonging to class I and II, respectively, in skeletal muscle of *S. tridecemlineatus* (Morin and Storey, 2006). Total HDAC activity was 1.82-fold higher in muscle of hibernating squirrels, compared with euthermic animals (Table 1). Furthermore, both HDAC1 and HDAC4 protein levels were elevated in hibernation by 1.2- and 1.5-fold, respectively, suggesting that enhanced synthesis of these enzymes is responsible, at least in part, for the overall elevation of HDAC during torpor. Although other HDACs should also be examined, both the increase in HDAC activity and in HDAC1 and HDAC4 protein content point to a regulated reduction in transcriptional activity during hibernation by targeted control of histones.

### *Regulation of RNA polymerase II*

RNA polymerase II is the enzyme that transcribes DNA to make mRNA. Previous studies showed that overall rates of transcript elongation were suppressed during hibernation, providing presumptive evidence that RNA polymerase II was suppressed. We directly measured RNA polymerase II activity by monitoring its ability to transcribe the gene for enhanced green fluorescent protein *in vitro* (Morin and Storey, 2006). Transcriptional activity in nuclear extracts from muscle of hibernating ground squirrels was only 57% of the value in euthermic muscle (assayed at 37°C) (Table 1). However, western blotting showed that total polymerase protein did not change during hibernation, which argues that the change in activity is the result of a stable modification of the enzyme. RNA polymerase II is known to be covalently modified with particularly prominent phosphorylation at Ser 2 and Ser 5 of a peptide sequence (YSPTSPS) that is repeated multiple times in the C terminal domain (CTD) (Kim et al., 1997), although the functional significance of phosphorylation at these sites is still debated. Using an antibody specific for the 5th phosphoserine residue in this sequence, we found that the amount of phosphorylated (Ser5) RNA polymerase II protein increased 1.79-fold in muscle from hibernating squirrels. This suggests a correlation between transcriptional repression and hyperphosphorylation of the CTD.

## Cell cycle arrest

A major energy expense for organisms is growth and development so, clearly, entry into a hypometabolic state should include an overall suppression of cell proliferation, although some features of development may continue (e.g. gonad maturation over the winter). The phenomenon of ‘diapause development’ is also seen in many insects, whereby a very slow program of change continues during diapause culminating, after a specified length of time, in the ability to break diapause in response to appropriate environmental cues (Ti et al., 2004). For example, a key marker of diapause development in silkmoth *Bombyx mori* embryos is the induction of the enzyme sorbitol dehydrogenase, which allows this polyol protectant to be cleared (Fujiwara et al., 2006). Embryonic diapause, which must include cell cycle arrest, actually occurs widely across phylogeny. Well-studied invertebrate models include the embryos of cold-hardy *B. mori* and the desiccation resistant embryos of brine shrimp *Artemia* sp. (Clegg, 2001), whereas diapause at the blastocyst stage occurs in many mammalian species and is responsible for the phenomenon of delayed implantation (Mead, 1993; Lopes et al., 2004).

However, to date cell cycle arrest as it relates to hypometabolism in non-embryonic organisms has received very little attention. This is a key area for future research because there are many types of proliferative cells (as well as whole organs such as liver or skin) that should predictably exit from the cell cycle during hypometabolism. In brief, the traditionally defined stages of the cell cycle are: (a) gap phase

1 (G1), when preparatory events for DNA replication are initiated (e.g. genes activated, necessary proteins synthesized), (b) S phase, when DNA replication occurs, (c) gap phase 2, (G2) when proteins needed for mitosis are accumulated, (d) M phase, during which mitosis occurs and including subphases of prophase, metaphase, anaphase, telophase and cytokinesis (Douglas and Haddad, 2003). After cytokinesis, cells either re-enter G1 or exit the cell cycle into G0 phase, where they typically differentiate or enter quiescence. One might think that cell cycle arrest in hypometabolism would simply be a matter of reversible exit into G0 phase, but new studies indicate much greater complexities and show that there is much to be explored.

Hypoxic/anoxic models provide some interesting insights. It is well known that hypoxia induces growth arrest and that this is mediated as an action of HIF-1. The mechanism in mammalian fibroblasts appears to involve the activation of $p21^{cip1}$, a key cyclin-dependent kinase inhibitor that controls the G1 checkpoint (Koshiji et al., 2004). However, HIF-1 action on $p21^{cip1}$ is not direct because this gene does not contain a hypoxia response element (HRE). Instead, HIF-1 counteracts the action of the mitogenic transcription factor Myc, which normally represses $p21^{cip1}$ expression. The situation is different, however, in the anoxia-tolerant nematode, *C. elegans*. Full anoxia exposure triggers entry into a reversible state of suspended animation that includes cessation of cell division, developmental progression, feeding and motility. HIF-1 deficient mutants survive just as well as the wild type, indicating that the mechanism involved is not HIF-1 dependent (Padilla et al., 2002). Studies with *Drosophila melanogaster* embryos found another set of mechanisms. Under severe hypoxia these cells arrested either in metaphase or pre-S phase (i.e. late G1) (Douglas et al., 2005). The metaphase arrest seemed to result from stabilization of cyclin A levels, which normally must degrade before cells can progress to anaphase. The pre-S block was linked with elevated levels of the transcription factor E2F1, which stimulates preparatory measures in G0 or G1 cells that are moving into a proliferative state. Again, E2F1 is normally degraded before the move into S phase. Whether this E2F1 mechanism might be interrelated with the $p21^{cip1}$ mechanism remains to be seen. To add to the complexity, embryos of *Drosophila*, *C. elegans*, zebrafish and brine shrimp each arrest at different stages in the cell cycle in response to oxygen deprivation (Douglas and Haddad, 2003). Hence, it is very likely that multiple mechanisms for cell cycle arrest have been developed that serve different life stages (e.g. embryo *versus* adult), different cell types and different species, and that unraveling the complexities of cell cycle arrest in hypometabolic states will prove to be very challenging.

Further insights into the possible regulatory mechanisms that could be involved come from a fascinating new study that used microarray transcriptional profiling to evaluate human fibroblasts making the transition into the quiescent state (Coller et al., 2006). Significantly, the gene expression profile was different depending on the stress used to trigger exit from the cell cycle (mitogen withdrawal, contact inhibition or loss of adhesion). However, a subset of genes whose specific expression in non-dividing cells proved to be signal-independent was identified and assigned to the quiescence program itself. These included genes that enforced the non-dividing state as well as others that ensured the reversibility of the cell cycle arrest by suppressing terminal differentiation. This study suggests an important principle for species that use reversible hypometabolism to escape intermittent environmental stress exposures. Normally proliferative cells in these organisms must not only be arrested but programs of terminal differentiation, which often take over after exit from the cell cycle, must be inhibited so that the cells have the option of returning from the quiescent state once the stress is removed.

## Gene expression

Although the net rate of protein synthesis is strongly suppressed in hypometabolic states, all organisms evaluated to date show specific upregulation of a small percentage of genes during metabolic depression. The capacity to identify these genes has exploded in recent years with the use of DNA microarray technology, which has allowed researchers to gain a broad overview of the responses to stress by thousands of genes, especially by using heterologous screening techniques (Eddy and Storey, 2002). A critical finding of these studies is the identification of genes belonging to many new categories of metabolic function that had not previously been considered to contribute to hypometabolism; for example, genes encoding serine protease inhibitors (serpins), various kinds of antioxidant enzymes, iron storage proteins and mitochondrial-encoded subunits of electron transport system (ETS) proteins are showing up repeatedly in systems of both aerobic and anoxic hypometabolism (reviewed in Storey, 2003; Storey, 2004b; Storey, 2006a; Storey, 2006b; Storey, 2006c). The breadth of genes that respond during hypometabolism is also impressive and demonstrates that entry into a hypometabolic state requires responses by a huge variety of cell functions. For example, genes that are upregulated during hibernation in ground squirrels include $\alpha_2$-macroglobulin in liver, moesin in intestine, isozyme 4 of pyruvate dehydrogenase kinase (PDK4) and pancreatic lipase in heart, isoforms of uncoupling proteins (UCPs) and fatty acid binding proteins (FABPs) in multiple tissues, muscle motor proteins, six kinds of membrane transporters in kidney, the melatonin receptor, eight types of serpins in multiple organs, HIF-1 in BAT and muscle, the transcription factors PPARγ and PGC-1α, carnitine palmitoyl transferase-1β, several antioxidant enzymes, iron-storage proteins, and four genes on the mitochondrial genome (Srere et al., 1995; Carey et al., 2003; Andrews, 2004; Eddy et al., 2005; Eddy et al., 2006; Morin and Storey, 2005; Storey, 2003; Storey, 2006b).

Interestingly, the types of genes that are upregulated in hypometabolic systems are also proving to be the same ones that underlie the phenomenon of ischemic preconditioning. Stenzel-Poore et al. (Stenzel-Poore et al., 2003) reported microarray analysis of the patterns of gene expression in a

mouse brain stroke model of injurious ischaemia *versus* preconditioning followed by injurious ischaemia (PII model). The patterns were strikingly different between the two and, notably, a pronounced downregulation of genes was observed in the PII model along with transcriptional changes that would suppress metabolic pathways and immune responses, reduce ion-channel activity, and decrease blood coagulation. All of these are hallmarks of natural hypometabolic states, which suggests that, with appropriate signalling (e.g. preconditioning), a program of metabolic suppression providing ischemia defense can be activated in mammalian models that are normally harmed by ischemia.

The upregulated genes identified to date in hypometabolic systems often fall into two general categories: (a) those that protect or preserve cellular metabolism and macromolecules under stress, and (b) those that address special needs for species-, tissue- or stress-specific functions during hypometabolism. Examples of the former include chaperone proteins, serpins, iron-storage proteins and antioxidant enzymes. Examples of the latter include enhancement of proteins and enzymes of fatty acid oxidation during mammalian hibernation, upregulation of urea cycle enzymes in estivating amphibians, increased expression of antifreeze proteins and/or enzymes involved in cryoprotectant synthesis in cold-hardy species, etc. Because normal routes of translation initiation (i.e. cap-dependent binding of the 5′end of mRNA with the eukaryotic initiation factor 4) are inhibited during dormancy by strong controls on ribosomal initiation and elongation factors, different mechanisms of translation initiation can be required to achieve the synthesis of stress-responsive genes (DeGracia et al., 2002). One of the ways of doing this is *via* a cap-independent mechanism involving an internal ribosome entry site in the 5′UTR of these genes. Approximately 3–5% of cellular mRNAs are translated in this manner, one of the most notable being the alpha subunit of HIF-1 (Holcik and Sonenberg, 2005).

Another way to achieve differential synthesis of stress-responsive proteins is to ensure that their mRNA transcripts remain associated with polysomes, the active translation machinery. Polysome dissociation has been widely documented in metabolic depression in situations as diverse as mammalian hibernation and anaerobiosis in brine shrimp and marine snails (reviewed by Storey and Storey, 2004). The effect is to permit the vast majority of mRNA transcripts to be retained during hypometabolism but sequestered into the translationally silent monosome fraction. However, transcripts of selected proteins whose expression is upregulated during hypometabolism remain in the polysome fraction; for example, this was seen for the H isoform of fatty-acid-binding protein in hibernator BAT and for the iron-binding protein, ferritin, in anoxia-tolerant marine snails (Hittel and Storey, 2002; Larade and Storey, 2004).

## Defense mechanisms for long-term viability

When endogenous fuel reserves are limiting and when dormancy must be sustained for an indeterminate length of time, mechanisms that prevent, minimize or reverse damage to macromolecules are key because dormant organisms cannot afford the high ATP expenditures required to repair, degrade or replace damaged cellular components. Furthermore, since hypometabolism is a defense against extreme environmental stress, even dormant organisms still need protection from the effects of external stresses, e.g. very high or very low temperatures, water limitation, oxygen free radicals, ionizing radiation, heavy metals, etc. One defense mechanism that has been extensively studied is the accumulation of low molecular mass metabolite protectants. Examples include the high levels of polyhydric alcohols (e.g. glycerol, sorbitol) that are used by cold-hardy insects for protection against freezing, the build-up of urea to provide desiccation resistance in water-stressed organisms, and the high trehalose and proline levels that are used to preserve membrane bilayer structure in anhydrobiotes (Clegg, 2001; Storey, 1997a; Storey, 2002).

As one result of advanced gene-screening technology, researchers are coming to realize that a variety of proteinaceous defenses are also critical for preserving viability and are another common element of hypometabolism across phylogeny. We will briefly consider three kinds here.

### *Antioxidant defenses*

Damage to cellular macromolecules by reactive oxygen species (e.g. superoxide, hydrogen peroxide, hydroxyl radical) is a fact of life and all organisms maintain antioxidant defenses that act at three levels: prevention, repair or disposal. Antioxidant defenses working at the prevention level have received the most attention by researchers in comparative biochemistry and include metabolites (e.g. ascorbate, vitamin E), peptides (glutathione, thioredoxin) and enzymes (e.g. superoxide dismutase, catalase, peroxiredoxin, glutathione peroxidase, glutathione *S*-transferase), as well as associated enzymes such as glutathione reductase and thioredoxin reductase that regenerate reduced peptides using the NADPH provided by G6PDH. Recent papers have documented the elevation of antioxidant defenses and/or upregulation of genes encoding antioxidant enzymes in association with hypometabolic excursions (for reviews, see Hermes-Lima et al., 2001; Hermes-Lima and Zenteno-Savin, 2002; Drew et al., 2002; Ramos-Vasconcelos and Hermes-Lima, 2003; Storey, 2004d; Storey, 2006a; Storey, 2007). Arousal from these states (e.g. hibernation, estivation, anaerobiosis) involves a large increase in oxygen uptake, concentration and consumption that causes a ‘burst’ of ROS generation and ROS-mediated damage during the recovery process. Indeed, reperfusion injuries caused by a sudden increase in ROS are a major contributor to the net damage caused by ischemic episodes in medical conditions such as heart attack and stroke. Two basic modes of adaptive defense appear to occur in animals faced with highly variable oxygen conditions. Some species maintain very high constitutive antioxidant defenses to deal with frequent situations of oxygen variation (e.g. antioxidant enzyme activities in organs of anoxia-tolerant freshwater turtles are

several-fold higher than other ectothermic vertebrates), whereas other species enhance defenses during the hypometabolic episode in preparation (or anticipation) of their immediate need during arousal/recovery (reviewed by Hermes-Lima et al., 1998; Hermes-Lima et al., 2001). Hibernating ground squirrels are a good illustration of the latter. Plasma ascorbate concentrations increase three- to fivefold during hibernation but fall rapidly during arousal with the highest rate of ascorbate use correlating with the peak of $O_2$ consumption during thermogenesis and with a transient peak in plasma urate (a degradation product of ROS attack) (Drew et al., 2002). Plasma ascorbate was also >threefold higher in plasma of hibernating *versus* aroused hamsters (*Mesocricetus auratus*) and ascorbate levels in brain extracellular fluid (ECF) fell markedly during arousal from hibernation and remained low throughout the interbout (Osborne and Hashimoto, 2006). Interestingly, ECF levels of reduced glutathione responded in the opposite way being very low in torpor but rising rapidly to high stable levels during interbout.

Another view of the situation in natural hypometabolic systems is also possible. This is the idea that because of metabolic suppression the capacity to replace/repair ROS-damaged macromolecules is low in the hypometabolic state. Hence, preventative defenses are the key to minimizing cumulative ROS damage over extended periods of dormancy. This idea fits well with new results from gene screening studies that show a widespread enhancement of another type of antioxidant defense in hypometabolic organisms – iron-binding proteins. We have found upregulation of genes including H and L chains of ferritin (the intracellular iron-storage protein) and the transferrin receptor (TfR2) that is responsible for uptake of iron-transferrin complexes into cells (transferrin being the plasma iron-binding protein) in diverse systems of hypometabolism, including during anoxia exposure in marine snails, anoxia or freezing exposure in hatchling turtles, and hibernation in bats (Larade and Storey, 2004; Storey, 2006a; Storey, 2006b). Indeed, ferritin mRNA levels were enriched in the polysome fraction under anoxia in marine snails, indicating that ferritin is one of the few actively translated proteins in the hypometabolic state. Iron is a vital component of many functional proteins in cells, but free iron in the ferrous state ($Fe^{2+}$) participates in the Fenton reaction with hydrogen peroxide and lipid peroxides to generate highly reactive hydroxyl radicals and lipid radicals (Hentze et al., 2004). Hence, long-term viability would be promoted by mechanisms that minimize iron-mediated ROS generation by maximizing the uptake and storage of iron in hypometabolic states (Storey, 2006a; Storey, 2006b).

### *Serpins*

Another prominent group of genes that we consistently find upregulated in hypometabolic systems are the serpins. To date, we have documented serpin upregulation in liver (Table 2) and selected other organs, in response to anoxic submergence of adult turtles, freezing or anoxia exposures in hatchling turtles, and hibernation in ground squirrels (Storey, 2004a; Storey, 2004c; Storey, 2006a). Serpins are a superfamily of proteins with 16 clades. Most are plasma proteins (typically secreted by liver) that act as irreversible covalent inhibitors of proteases that cleave specific proteins. Many inhibit the proteases that act at critical checkpoints in self-perpetuating proteolytic cascades such as the proteases involved in blood coagulation, fibrinolysis, inflammation and complement activation (Gettins, 2002). A role for serpins in hypometabolism would seem to make sense because elevation of selected serpins could be key to controlling various proteolytic reactions and cascades that

Table 2. *Proteinase inhibitors identified from cDNA array screening as putatively upregulated (>twofold increase in expression levels) in liver of different species during hypometabolism*

| Inhibitor | Full name | Function | Situation | Species | Reference |
|---|---|---|---|---|---|
| SERPINA1 | $\alpha_1$-antitrypsin | Inhibits elastase, trypsin, chymotrypsin, thrombin, plasmin, kallikrein, collagenase | Hibernation | Ground squirrel | (Storey, 2004c) |
| SERPINA7 | Thyroxin-binding globulin | Plasma transport of thyroid hormones | Hibernation | Ground squirrel | (Storey, 2004c) |
| SERPINB9 | Cytoplasmic antiprotease 3 | Inhibits caspase-1, granzyme B | Hibernation | Ground squirrel | (Storey, 2004c) |
| SERPINC1 | Antithrombin | Inhibits thrombin, factor Xa, IXa | Anoxia | Red-eared slider | (Storey, 2004a) |
| | | | Freezing and anoxia | Painted turtle | (Storey, 2006a) |
| | | | Hibernation | Ground squirrel | (Storey, 2004c) |
| SERPIND1 | Heparin cofactor II | Inhibits thrombin | Anoxia | Red-eared slider | (Storey, 2004a) |
| | | | Freezing and anoxia | Painted turtle | (Storey, 2006a) |
| SERPINF1 | Pigment epithelium derived factor (PEDF) | Non-inhibitory towards proteinases; acts as an anti-angiogenic factor | Anoxia | Red-eared slider | (Storey, 2004a) |

The species analyzed were thirteen-lined ground squirrels *Spermophilus tridecemlineatus*, adult red-eared slider turtles *Trachemys scripta elegans*, and hatchling painted turtles *Chrysemys picta marginata*. Note that the serpin B clade are intracellular protease inhibitors and selected serpins such as A7 and F1 have lost their protease functions and taken on new actions.

might otherwise cause cumulative damage to tissues over long-term dormancy.

Some specific actions by individual serpins during hypometabolism can be envisioned, although it must be noted that none of these ideas has yet received in depth analysis. SERPINA1 ($\alpha_1$-proteinase inhibitor or $\alpha_1$-antitrypsin) is the most abundant of the circulating serpins; its main physiological role is to protect the lower respiratory tract from proteolytic destruction by neutrophil elastase, which hydrolyzes structural proteins as part of its attack on bacterial infections (Kalsheker et al., 2002). Hibernation-responsive upregulation of SERPINA1 might act to suppress elastase activity under the conditions of low body temperature and apnoic breathing patterns during torpor, which should be associated with reduced intake of air-borne pathogens. This could help to avoid non-specific damage to tissues over what could be days or weeks of torpor when cellular repair and proliferation functions are suppressed. It will be interesting to trace the changes in SERPINA1 levels over the course of torpor/arousal bouts to see if its levels mirror changes in ventilatory flow rates. SERPINC1 (antithrombin) and SERPIND1 (heparin cofactor II) both inhibit thrombin to block the clotting cascade (Gettins, 2002). A consistent characteristic of hypometabolism is bradycardia, which means low blood flow conditions and an increased risk of thrombosis (spontaneous clot formation) in the microvasculature. This could be countered by upregulation of these two serpins (Table 2). Indeed, other mechanisms of clotting inhibition are also known for mammalian hibernation, including elevation of plasma $\alpha_2$-macroglobulin (a non-serpin inhibitor of clotting cascade proteases) (Srere et al., 1995) as well as reduced levels of platelets and several clotting factors (McCarron et al., 2001). Taken together, these emphasize the importance of thrombosis prevention in the torpid animal.

SERPINF1, also known as pigment epithelium-derived factor (PEDF), is a protein with an intriguing function that we found upregulated in response to anoxia in turtles (Table 2). This serpin does not inhibit a plasma proteinase but instead has potent anti-angiogenic and neurotrophic actions and counteracts the angiogenic effects of vascular endothelial growth factor (VEGF) (Duh et al., 2002; Petersen et al., 2003). VEGF stimulates the proliferation of blood vessels and is regulated by HIF-1 in response to hypoxia stress. PEDF was discovered as the factor that inhibited aberrant blood vessel growth in models of ischemia-induced retinopathy (Stellmach et al., 2001) and is now known to act in other tissues (Doll et al., 2003). Indeed, plasma levels of PEDF are high enough to suggest that systemic delivery of the protein could affect angiogenesis throughout the body (Petersen et al., 2003). Upregulation of PEDF in anoxia-tolerant species is intriguing because these animals have something of a conundrum. In some situations they need to respond to low oxygen availability with improved oxygen delivery to tissues (e.g. during growth and development of tissues or when exposed to persistent hypoxia) using conserved vertebrate mechanisms of HIF-1 activation of VEGF (to stimulate angiogenesis) and erythropoietin (to synthesize more red blood cells). In situations of breath-hold submergence, however, it is counter-intuitive to enhance capillary growth since the problem is not oxygen delivery but a lack of oxygen overall. Hence, upregulation of PEDF could counteract a VEGF-mediated angiogenic response in these situations. Future analysis of PEDF actions in anoxia-tolerant *versus* -intolerant species will be very interesting.

### *Chaperone proteins*

Chaperones are proteins that bind to and stabilize an otherwise unstable conformer of another protein and, by controlled binding and release of the substrate protein, facilitate its correct folding, oligomeric assembly, or transport to a particular subcellular compartment (Hendrick and Hartl, 1993). Many types of chaperones are constitutive in cells but the amounts and/or types of chaperones are also enhanced in response to many kinds of environmental and endogenous stresses that destabilize protein structure/function and lead to the unfolding or aggregation of existing proteins or the malfolding of nascent proteins during their synthesis (Goldberg, 2003). Indeed, a common response to many kinds of stress is the suppression of general protein synthesis and the activation instead of shock proteins that then act to rescue cellular proteins from the effects of the stress. Various studies have confirmed that synthesis of shock proteins is a component of natural hypometabolism. Elevated levels of chaperones in hypometabolic states can provide long-term conformational stability to cellular proteins to defend against environmental insults occurring while the organism is dormant, and to extend the 'life-span' of proteins that might normally undergo high turnover but that cannot be easily replaced under the energy constraints of the hypometabolic state.

One of the first shock proteins to be associated with long-term hypometabolism was p26, a small heat shock/alpha-crystallin protein that accumulates in high levels in encysted brine shrimp embryos (Willsie and Clegg, 2001; MacRae, 2003). Although exhibiting chaperone activity, p26 also migrates to the nucleus under heat or anoxia stress and seems have a key action in transcriptional repression. Accumulation of heat shock proteins (hsp23, hsp70) has also been documented in response to desiccation in nondiapausing pupae of the flesh fly and these proteins also build up to high levels as an early response when larvae enter diapause (Hayward et al., 2004; Hayward et al., 2005). Array screening of turtle brain for anoxia-responsive proteins also highlighted the putative upregulation of several shock proteins. These were HSP70-1A (the inducible form, also known as Hsp72), HSP70-9B (also known as mortalin-2 or Grp75), HSP40 (also known as DnaJ or HSJ1), and αB-crystallin (Storey, 2007). Elevated levels of Hsp72 protein have been reported previously as a response to anoxia in turtle brain and other organs (Prentice et al., 2004; Ramaglia and Buck, 2004). HSP40 is the co-chaperone of HSP70 that acts as a 'holdase' in partnership with the ATP-dependent HSP70 'foldase' (Winter and Jakob, 2004), so it is not surprising that they are co-ordinately expressed under anoxia.

HSP70-9B or mortalin-2 is intriguing. It is found predominantly in mitochondria but, depending on its subcellular niche and binding partner, it performs multiple functions relevant to cell survival, control of proliferation and stress response. Of particular interest to hypometabolism is its role in lifespan extension; for example, its induction by heat stress or transgenic overexpression increased nematode lifespan by >40% (Yokoyama et al., 2002), apparently due to an ability to inactivate the tumor suppressor protein p53 (Wadhwa et al., 2002). This protein clearly deserves more attention to assess its expression and role in multiple forms of hypometabolism.

We have recently begun to assess the role of another type of chaperone in hypometabolic states. These are the endoplasmic reticulum (ER) resident chaperones, in particular the glucose-regulated protein, GRP78. We analyzed GRP78 expression in organs of hibernating ground squirrels *S. tridecemlineatus*, assessing both transcriptional and translational controls. Transcript levels of *grp78* were strongly increased by 3.5-fold in brown adipose tissue (BAT) and 4.1-fold in brain of hibernating squirrels, as compared with euthermic controls, but remained stable in other tissues tested (heart, kidney, liver, lung, skeletal muscle) (Mamady and Storey, 2006). Similarly, GRP78 protein was elevated in BAT and brain during hibernation, by 1.57- and 1.37-fold, respectively, but unaltered (kidney, muscle, lung) or lowered (heart and liver, by 19 and 42%, respectively). How can this be interpreted? BAT and brain are both highly oxygen-dependent organs and they are both organs that need to retain considerable metabolic activity during torpor. BAT provides low level heating to keep body tissues from freezing if ambient temperature falls below 0°C and BAT is also the organ that powers arousal *via* nonshivering thermogenesis. Protein biosynthetic capacity remains uninhibited in BAT (although rates would be reduced at low body temperatures) (Hittel and Storey, 2002) and, even in deep torpor, BAT can synthesize more uncoupling protein if there is a need to enhance thermogenic capacity (Boyer et al., 1998; Barger et al., 2006). Brain must retain its sensing and signalling capacity during torpor in order to determine when to trigger arousals. Thus, these two organs may have the greatest need for protein synthesizing capacity during both torpor and arousal. Hence, elevated levels of ER chaperones make sense. GRP78 has a central role in the folding and assembly of proteins in the ER and is also a sensitive biomarker of the unfolded protein response (UPR) (Lee, 2001). Stress conditions that perturb ER function or cause the accumulation of malfolded proteins trigger the UPR, which then acts to help cells recover from the stress condition (Schröder and Kaufman, 2005). The UPR includes enhanced synthesis of GRP78 to help rectify the problem of high levels of unfolded proteins but GRP78 also has other actions. One of these is apoptosis inhibition by interacting with caspase-7 and caspase-12 to block their activation. Hence, GRP78 action can both guide the correct folding of the selected proteins that need to be made in the hypometabolic state as well as counteract events that would otherwise lead to apoptosis in cells experiencing unusual stress conditions.

## Conclusions

The present review has surveyed many of the current ideas about how hypometabolism is regulated. Two main conclusions can be drawn. Firstly, the principles of metabolic regulation of hypometabolism are highly conserved across phylogeny, indicating that they are ancient mechanisms derived from ancestral cells. Hence, it is reasonable to propose that the mechanisms are perhaps present in all species but latent in those that have abandoned hypometabolism (e.g. humans) as a defense strategy against environmental insult. Thus, the goal of some researchers to find mechanisms to induce and control hypometabolism as a medical intervention strategy seems justified. Secondly, it is clear that hypometabolism is a multi-faceted process requiring regulation and coordination of virtually all aspects of metabolism. Much more remains to be determined and many key areas are still largely unexplored in hypometabolic systems, including the regulation of protein degradation, control of the cell cycle, the prevention of apoptosis and atrophy during long-term torpor, and the possible central role of mitochondria in suppressing metabolic rate.

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