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# Metabolic suppression in mammalian hibernation: the role of mitochondria.

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COMMENTARY

# Metabolic suppression in mammalian hibernation: the role of mitochondria

**James F. Staples***

## ABSTRACT

Hibernation evolved in some small mammals that live in cold environments, presumably to conserve energy when food supplies are low. Throughout the winter, hibernators cycle spontaneously between torpor, with low metabolism and near-freezing body temperatures, and euthermia, with high metabolism and body temperatures near 37°C. Understanding the mechanisms underlying this natural model of extreme metabolic plasticity is important for fundamental and applied science. During entrance into torpor, reductions in metabolic rate begin before body temperatures fall, even when thermogenesis is not active, suggesting active mechanisms of metabolic suppression, rather than passive thermal effects. Mitochondrial respiration is suppressed during torpor, especially when measured in liver mitochondria fuelled with succinate at 37°C *in vitro*. This suppression of mitochondrial metabolism appears to be invoked quickly during entrance into torpor when body temperature is high, but is reversed slowly during arousal when body temperature is low. This pattern may reflect body temperature-sensitive, enzyme-mediated post-translational modifications of oxidative phosphorylation complexes, for instance by phosphorylation or acetylation.

**KEY WORDS: Heat, Body temperature, Thermoregulation, Thermogenesis, Oxidative phosphorylation, Post-translational modification, Acetylation, Phosphorylation**

## Introduction

Most mammals are strict endotherms, i.e. they maintain fairly constant body temperatures ($T_b$) near 37°C using heat derived primarily from endogenous metabolism. In cold environments, mammals retain some of this heat by regulating insulation (using underfur and/or subcutaneous fat), peripheral blood circulation (using vasoconstriction and/or countercurrent heat exchangers) and ventilatory evaporation. At very cold temperatures, the high gradient between $T_b$ and ambient temperature ($T_a$) causes large heat loss by radiation and conduction, which is also affected by convection of water or air. For small mammals, the high body surface area, relative to volume, results in greater mass-specific rates of heat loss. While large land mammals can increase insulation by changing the quality (microstructure) and quantity (length, density) of underfur, this option is limited for small mammals. Imagine a 70 mm long lemming growing fur 100 mm long; it might stay warm, but when it tripped over that fur it would be easy prey.

Despite the challenges, many small mammals thrive in cold environments. To compensate for high heat loss, these mammals upregulate their capacities to produce metabolic heat. Though effective, this strategy requires large quantities of fuel during winter when food availability is typically low. Many small mammals use stored food to power thermogenic metabolism. For example, American red squirrels (*Tamiascurius hudsonicus*) cache spruce cones and feed on the seeds throughout winter, allowing them to maintain $T_b$ near 37°C. Other small mammals, such as the arctic ground squirrel (*Urocitellus parryii*), evolved a completely different strategy. These hibernators reduce metabolic rate (MR; typically measured as oxygen consumption) by over 90% and permit $T_b$ to fall as low as −2.9°C (Barnes, 1989), allowing them to survive the cold winter solely on energy stored within their bodies.

To illustrate a point, in the preceding paragraph I used obvious examples of animals from sub-polar and polar regions that do and do not (cannot?) hibernate. Mammalian species, however, exhibit a continuum of metabolic and thermoregulatory phenotypes, ranging from strict endotherm to obligate hibernator, in many habitats. A specific definition of hibernation is a matter of seemingly endless debate among biologists, but most agree that it involves significant suppression of MR and lowering of $T_b$ for periods of several days during the winter. Similar phenomena include daily torpor, which involves shorter (<24 h), less intense drops in $T_b$ and metabolism, and estivation, which typically occurs in summer.

Hibernation and similar phenotypes are found in most mammalian and some bird orders, including tropical and subtropical primates (Daussman et al., 2012). Within phylogenetic groups, however, the distribution of the hibernation phenotype is quite broad. For example, three species of sciurid rodent (squirrel family, order Rodentia) share the habitat of my temperate (42.98ºN), southern Ontario garden. One is an obligate hibernator (groundhog, *Marmota monax*), another is a facultative hibernator (eastern chipmunk, *Tamius striatus*) and the third a strict endotherm (gray squirrel, *Sciurius carolinensis*). Within this family the hibernation phenotype may relate to circannual fluctuations of a specific blood protein complex (Kondo et al., 2006; Sekijima et al., 2012). These findings suggest the existence of a 'hibernation induction trigger', though they would benefit from rigorous independent confirmation. Moreover, comparisons with other mammalian hibernators could provide valuable information regarding the potential function of this protein complex and the evolution of hibernation, a subject beyond the scope of this review [interested readers should consult Geiser (Geiser, 1998)]. While hibernation clearly depends on surviving low $T_b$ that would kill most mammals, this review focuses on current knowledge about how hibernators reduce metabolism in order to conserve energy.

## Hibernation patterns

Most biologists classify a mammal as a hibernator if its $T_b$ falls below 10°C and its MR falls by over 90% for longer than a day (Geiser, 2011). Unlike hypothermia, the decrease in $T_b$ is controlled and regulated, and hibernators can spontaneously re-warm using solely endogenous metabolic heat. Recovery from hypothermia

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## Glossary

**Electron transport system (ETS)**
A series of enzymes associated with the inner membrane of mitochondria that catalyze the transfer of electrons derived from the oxidation of energetic substrates. Each electron transfer releases free energy (and heat) which is used to pump protons from the mitochondrial matrix to the space between the inner and outer mitochondrial membranes, thereby generating a proton gradient. This gradient is used to synthesize ATP, which can be used to power energetic reactions in the cell.

**Interbout euthermia (IBE)**
The period within the torpor bout during which metabolic rate and body temperature are sustained at high levels for several hours. In small hibernators these periods occur regularly throughout the hibernation season and are separated by several days of torpor.

**State 3 respiration**
A near-maximal state of mitochondrial oxygen consumption and ATP synthesis, measured in the presence of saturating concentrations of oxygen, energetic substrates and ADP.

**Thermoregulatory set point ($T_{set}$)**
The body temperature below which endotherms activate mechanisms of heat conservation and/or metabolic heat generation. It is analogous to the temperature to which a furnace is set. In mammals, $T_{set}$ is determined by neurons within the pre-optic anterior hypothalamus of the brain.

**Uncoupling protein 1 (UCP1)**
A protein found only in the inner mitochondrial membrane of brown adipose cells of mammals allowing them to produce metabolic heat for the regulation of body temperature. When these cells are stimulated by norepinephrine, UCP1 permits protons to leak back into the mitochondrial matrix. This leak activates the ETS to transport more electrons, releasing more heat, but without a proton gradient, no ATP is synthesized.

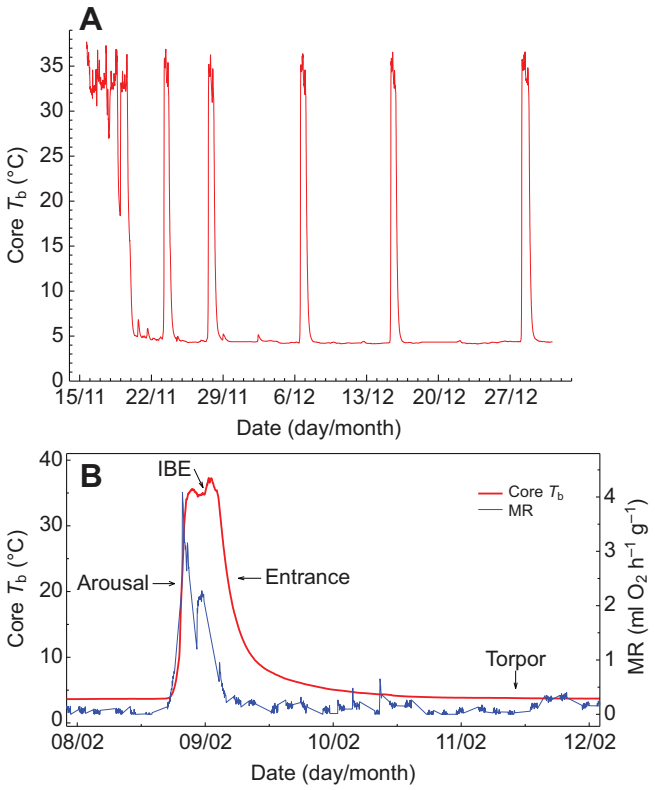


**Fig. 1. Body temperature and metabolic rate in a hibernator.** (A) Body temperature ($T_b$) of a 13-lined ground squirrel at the beginning of the hibernation season. (B) Metabolic rate (MR) and $T_b$ of a ground squirrel in different stages of a torpor bout. IBE, interbout euthermia.

requires an exogenous heat source, but hibernators in the wild will sometimes supplement endogenous heat production with exogenous heat. Obligate hibernators apparently follow an endogenous circannual rhythm, and 'must' hibernate each year, regardless of environmental conditions. This fact is dramatically illustrated by Pengelley and colleagues' research, which showed that golden-mantled ground squirrels (*Callospermophilus lateralis*), born in captivity and maintained under constant light and temperature, hibernated each year, close to when winter would begin in the wild (Pengelley et al., 1976). Facultative hibernators, such as the Syrian hamster (*Mesocricetus auratus*), can hibernate at any time of the year, but require acclimation for several weeks to cold temperatures. Photoperiod, food availability and food quality, especially polyunsaturated fats (Harlow and Frank, 2001), can also affect facultative hibernation.

As autumn progresses, obligate hibernators, such as ground squirrels, undergo a series of discrete hibernation bouts that can be divided into four stages: (1) entrance, where MR falls rapidly by over 90%, and $T_b$ falls subsequently towards $T_a$; (2) torpor, where MR and $T_b$ remain low and constant for several days; (3) arousal, where MR spontaneously increases rapidly over a few hours and $T_b$ rises to ca. 37°C; and (4) interbout euthermia (IBE), where MR and $T_b$ remain high and constant for several hours before a new bout begins (Fig. 1). The duration of these phases depends on body mass within (Zervanos et al., 2013) and among species (French, 1985). The reduction in MR and $T_b$ during entrance and torpor undoubtedly conserves energy, but arousal and IBE are quite expensive. In Richardson's ground squirrels (*Urocitellus richardsonii*), up to 88% of the energy expended over the hibernation season may be used during arousal and IBE (Wang and Wolowyk, 1988), so these phases are assumed to be important. Many things change during arousal and IBE, including gene expression, protein synthesis and gluconeogenesis (Carey et al., 2003), and some hibernators appear to spend much of the IBE in non-REM sleep (Daan et al., 1991). However, we do not yet know why, or even if, arousals are necessary, nor do we fully understand how they are controlled.

The definition of hibernation used at the beginning of this section is useful for discussion but excludes some mammals that many people would consider hibernators. Black bears (*Ursus americanus*) pass most of the winter in underground dens, without eating or drinking, but $T_b$ falls only to 30°C (Tøien et al., 2011). In fact, no mammal larger than ca. 10 kg is known to reach the low $T_b$ of 'true' or 'deep' hibernators. Despite the modest drop in $T_b$, bears reduce MR by as much as 75% below basal levels (Tøien et al., 2011). As a biochemist, I find this degree of metabolic suppression impressive, so I consider bears to be hibernators!

## Temperature, heat and metabolism

Decreases in temperature reduce the rate of enzyme-catalyzed reactions (the so-called Arrhenius or $Q_{10}$ effect) (Hochachka and Somero, 2002), so in hibernators the drop in $T_b$ during entrance, on its own, will reduce MR passively. However, the drop in MR precedes the drop in $T_b$, suggesting an active, regulated metabolic suppression. This initial drop in MR is probably due mostly to coordinated changes in thermoregulation. In mammals, $T_b$ is regulated by neurons in the pre-optic anterior hypothalamus that establish the thermoregulatory set-point ($T_{set}$). If $T_b$ falls below $T_{set}$, heat conservation mechanisms are activated. These mechanisms,

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which include piloerection (‘fluffing up’ of fur) and peripheral vasoconstriction, are effective and require little energy, but if low $T_a$ drives $T_b$ even lower, thermogenic metabolism is activated. In small hibernators during IBE, $T_{set}$ is ca. 37°C and $T_a$ is ca. 5°C, so thermogenesis is active and MR is high. During entrance and torpor, $T_{set}$ decreases (Heller et al., 1977), thermogenesis stops and MR decreases. Hibernators regulate $T_b$ even during torpor, and if $T_a$ drops below the already low $T_{set}$, thermogenesis is activated and $T_b$ is defended (Buck and Barnes, 2000).

Most thermogenic heat is derived from mitochondrial processes. Oxidation of NADH to $NAD^+$ by the electron transport system (ETS) releases 56.2 kcal $mol^{-1}$ of free energy (under standard conditions) but only ca. 40% of this energy is used to phosphorylate ADP to ATP. The rest is released as heat. All mitochondrial metabolism produces heat, but at low $T_a$ endotherms activate specific thermogenic mechanisms that do little useful work, but generate heat to regulate $T_b$. Mammalian thermogenesis is either coupled to or uncoupled from oxidative phosphorylation. Coupled thermogenesis can involve shivering and futile cycling of ions across membranes, both of which increase the rate of ATP hydrolysis. Hydrolysis of ATP does release some heat but its greatest thermogenic effect results from production of the hydrolytic product ADP. Binding of ADP to the mitochondrial $F_1F_O$ATPase (ETS complex V) allows protons to flow into the mitochondrial matrix, stimulating oxidative phosphorylation and flux through the ETS and thereby increasing heat production. Uncoupled thermogenesis does not depend on ADP and is best described in small eutherian mammals that possess brown adipose tissue (BAT). This unique tissue has high levels of mitochondria that contain very little $F_1F_O$ATPase but considerable amounts of uncoupling protein 1 (UCP1) (Fig. 2). When activated by sympathetic stimulation, UCP1 allows protons to flow into the mitochondrial matrix, stimulating ETS flux and heat production, but with virtually no ATP synthesis (Klingenspor and Fromme, 2012).

So, in hibernators it might be reasonable to hypothesize that initial energy savings are realized simply by reducing $T_{set}$ and thermogenesis during entrance, with the Arrhenius effect further reducing MR as $T_b$ falls. However, data do not support this hypothesis. The edible dormouse, *Glis glis*, will enter hibernation even at thermoneutral temperatures ($T_a$=28.6°C) when thermogenesis would not be active. Under these conditions, MR still decreases considerably during entrance before $T_b$ falls, suggesting that other mechanisms of metabolic suppression are invoked (Heldmaier et al., 2004).

## Mitochondria and hibernation

As the main sites of oxygen consumption and heat production, mitochondria have been studied for decades in hibernators (for review, see Staples and Brown, 2008). Designing hibernation experiments is inherently complicated because it is not always clear what conditions should be compared. For example, many early metabolic studies compared animals in torpor with summer euthermic ones. While MR and $T_b$ certainly differed between these groups, many other conditions also differed including $T_a$, photoperiod and feeding. Recent studies have attempted to control these variables by including comparison groups within the winter hibernation season (e.g. Nelson et al., 2009). In the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) we found modest (ca. 30%) suppression of mitochondrial respiration (under near-maximal ‘state 3’ conditions; see Glossary) in skeletal (Brown et al., 2012) and cardiac muscle (Brown et al., 2014) during torpor, but brain cortex mitochondria exhibit no apparent suppression (Gallagher and Staples, 2013). In contrast, mitochondria isolated from the liver of torpid ground squirrels exhibit state 3 respiration up to 70% lower than those from animals in IBE or summer conditions (Muleme et al., 2006; Brown et al., 2013). A similar, if less extreme, pattern is seen in mitochondria isolated from animals that undergo daily torpor (Brown et al., 2007), though it may be limited to specific tissues, especially the liver (Kutschke et al., 2013). This similarity raises the possibility that all forms of mammalian hypometabolism share common mechanisms and, perhaps, evolutionary origins.

The impressive degree of suppression in hibernation depends on experimental conditions, being greatest with succinate as a substrate. Succinate metabolism is relatively simple, requiring only transport across the inner mitochondrial membrane and oxidation by ETS complex II. Metabolism of pyruvate or fatty acid derivatives is more complex, and includes oxidation through the Krebs cycle. Suppression of liver mitochondrial respiration in torpor is more modest with these substrates, suggesting that much of the suppression occurs at or downstream of complex II.

Experimental temperature also affects mitochondrial metabolic suppression in torpor. This observation exemplifies the complexity of designing hibernation experiments; liver mitochondria from IBE ground squirrels were at ca. 37°C in their ‘native’ state before isolation, while those from torpid animals were at ca. 5°C. So at what *in vitro* temperature should one measure respiration? Using a range of temperatures, we found the greatest suppression at 37°C. At 25°C, suppression was still significant in torpor but more modest; however, at 10°C, torpor could not be statistically distinguished from IBE (Fig. 3) (Brown et al., 2012). These findings suggest that

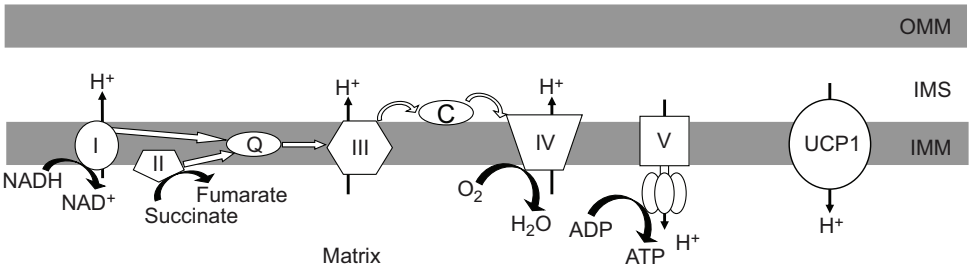


**Fig. 2. Typical animal mitochondrial bioenergetics.** The electron transport system (ETS) enzyme complexes associate with the inner mitochondrial membrane (IMM). NADH is oxidized by complex I and succinate by complex II. Electrons from these substrates are transferred to the mobile carrier coenzyme Q (Q), which transfers them to complex III, and subsequently to complex IV via cytochrome *c* (C). Approximately 40% of free energy released by substrate oxidation is used by complexes I, III and IV to pump protons from the matrix to the intermembrane space (IMS), between the IMM and the outer mitochondrial membrane (OMM). The remainder of the free energy is released as heat. The IMM of mammalian brown adipose tissue contains little complex V but, uniquely, significant amounts of uncoupling protein 1 (UCP1). When activated, protons flow from the IMS through UCP1 into the matrix, stimulating ETS substrate oxidation and heat production, but no ATP synthesis.

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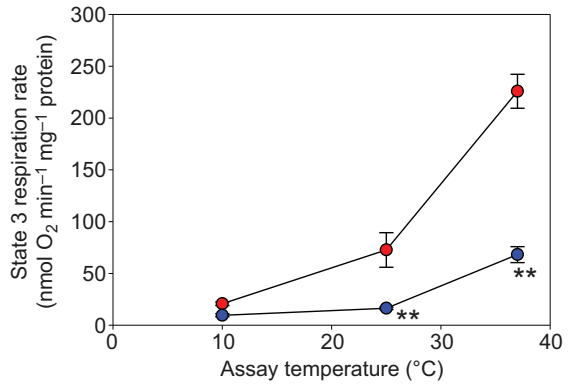


**Fig. 3. Temperature dependence of mitochondrial respiration.** Liver mitochondria were isolated from torpid (blue) and IBE (red) ground squirrels. State 3 respiration was measured with succinate as substrate (significant difference indicated by **). Adapted from Brown et al. (Brown et al., 2012).

active suppression of mitochondrial metabolism may have a greater impact on whole-animal MR in the initial stages of entrance, before $T_b$ falls substantially, so we investigated mitochondrial metabolism throughout a torpor bout. Respiration increases ca. 2-fold between torpor ($T_b$ 5°C) and early arousal ($T_b$ 15°C), and another 2-fold between early and late arousal ($T_b$ 30°C). Respiration does not peak until $T_b$ reaches ca. 37°C in IBE (Armstrong and Staples, 2010). In contrast, respiration is suppressed rapidly during entrance; between IBE and early entrance ($T_b$ 30°C), respiration falls by 70% and does not differ from that at late entrance ($T_b$ 15°C) or torpor (Chung et al., 2011) (Fig. 4). These data suggest that mitochondrial metabolism is suppressed by active mechanisms early in entrance.

## Mechanisms of metabolic suppression

The changes in mitochondrial respiration throughout a torpor bout suggest mechanisms that might underlie them. The expression of many genes changes throughout hibernation bouts (Hittel and Storey, 2002), contributing to many proteomic changes (Epperson et al., 2010), but these are unlikely to explain all of the observed changes in mitochondrial metabolism. Mitochondrial respiration appears to be suppressed quite quickly in entrance, probably faster than changes in transcription and translation occur. Moreover, peptide elongation in hibernators ceases below 18°C (van Breukelen and Martin, 2001) but in early arousal, when $T_b$ is much lower, mitochondrial respiration increases significantly. Our search for mechanisms, therefore, has focused on acute regulation of pre-existing proteins.

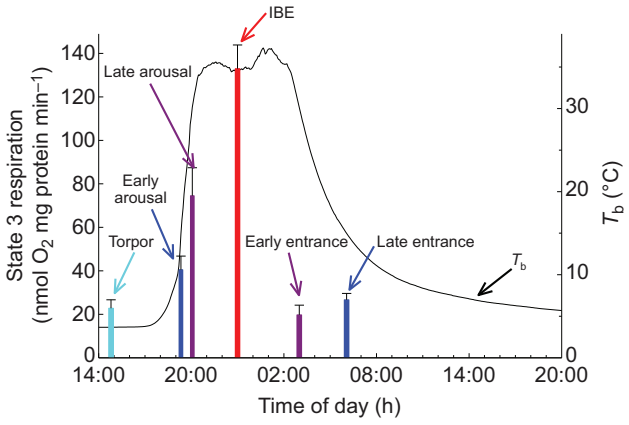


**Fig. 4. The dynamics of mitochondrial metabolism throughout a torpor bout.** Succinate-fuelled state 3 respiration, measured at 37°C, of liver mitochondria sampled from 13-lined ground squirrels in torpor, early and late arousal, IBE, and early and late entrance. Data, adapted from previous studies (Armstrong and Staples, 2010; Chung et al., 2011), are presented against a representative trace of core $T_b$.

Succinate is transported across the inner mitochondrial membrane by the dicarboxylate transporter, so the observed suppression of succinate respiration may be caused simply by inhibition of this transporter. The dicarboxylate transporter may be inhibited by excesses of co-enzyme A conjugates of long-chain fatty acids, and this inhibition can be relieved by the addition of carnitine. However, we found no evidence that succinate transport is differentially regulated in liver mitochondria from torpid versus IBE ground squirrels (Cooper et al., 2014). Moreover, the apparent affinity for succinate oxidation by intact mitochondria does not differ between these two conditions, arguing against changes in the kinetics of succinate transport (Brown et al., 2013).

Metabolomic changes do occur between torpor and IBE (Nelson et al., 2009; Nelson et al., 2010), and ETS complex II is inhibited by oxaloacetate, a Krebs cycle intermediate. We found that complex II was indeed inhibited by oxaloacetate in torpor and early arousal, but relief of this inhibition (by preincubation with isocitrate) did not fully 'rescue' state 3 respiration to IBE levels. At most, metabolite inhibition of complex II can account for 25% of this suppression (Armstrong and Staples, 2010).

## A role for post-translational modifications?

The dynamics of mitochondrial respiration throughout a torpor bout points to a temperature-sensitive mechanism – suppression occurs rapidly during entrance when $T_b$ is fairly high but is reversed only slowly during arousal when $T_b$ starts at low levels. Enzymes that covalently modify other enzymes, for example by phosphorylation or acetylation, could account for this pattern. Indeed, muscle phosphoglucomutase, a cytosolic enzyme, is differentially phosphorylated among hibernation states (Hindle et al., 2011).

Within mitochondria, soluble adenylate cyclase (sAC) can be activated by changes in the content of ATP, $Ca^{2+}$ and $HCO_3^-$ (Valsecchi et al., 2013). Activation of sAC would stimulate intramitochondrial protein kinase A (PKA) (Schwoch et al., 1990), which can phosphorylate many ETS proteins (Valsecchi et al., 2013), altering their activity (Lee et al., 2005; Tomitsuka et al., 2009; Phillips et al., 2012). Because sAC and PKA are temperature sensitive, such a mechanism could explain the pattern of mitochondrial suppression in hibernators, but to date no changes in the phosphorylation of mitochondrial proteins have been found among torpor bout phases, though seasonal differences are evident (Chung et al., 2013).

Three protein deacetylases, SIRT3, SIRT4 and SIRT5, are found within the matrix of mammalian mitochondria (Anderson and Hirschey, 2012). Fasting increases liver mitochondrial SIRT3 expression, affecting oxidative metabolism (Hirschey et al., 2010). SIRT3-mediated deacetylation alters ETS complex II activity (Cimen et al., 2010), a result that is particularly relevant to hibernators given the 70% suppression of succinate oxidation in torpor. To my knowledge, however, no studies have sought differential acetylation of mitochondrial proteins among torpor stages.

Obviously dephosphorylation and acetylation may also play roles in regulating mitochondrial metabolism in hibernation, but I know of no studies that demonstrate intramitochondrial acetylase or phosphatase activities. Moreover, I know of no studies that have examined other forms of post-translational modifications, such as *S*-nitrosylation, nitration and glutathionylation, in hibernation.

The ability of hibernators to cycle between the typical pattern of high MR and warm $T_b$ to one of low metabolism and near-freezing $T_b$ represents an inherently fascinating natural phenomenon. Besides inspiring curiosity, hibernation may represent an ancestral condition, so understanding such metabolic plasticity may inform research on mammalian evolution. Understanding the regulation between ATP supply and demand is important for understanding some pathological conditions (Covian and Balaban, 2012), and hibernators offer a natural model with insights not available from traditional mammalian models. Hibernators also appear to be resistant to ischemia/reperfusion injury (Lindell et al., 2005; Dave et al., 2006) and the ability to reversibly suppress oxidative metabolism is probably key to this resistance. For all of these reasons, the study of the reversible suppression of mitochondrial metabolism in mammalian hibernators promises to advance both fundamental and applied science for years to come.

**Competing interests**
The author declares no competing financial interests.

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