Metabolic Flexibility: Hibernation, Torpor, and Estivation

James F. Staples[*1]

ABSTRACT

Many environmental conditions can constrain the ability of animals to obtain sufficient food energy, or transform that food energy into useful chemical forms. To survive extended periods under such conditions animals must suppress metabolic rate to conserve energy, water, or oxygen. Amongst small endotherms, this metabolic suppression is accompanied by and, in some cases, facilitated by a decrease in core body temperature—hibernation or daily torpor—though significant metabolic suppression can be achieved even with only modest cooling. Within some ectotherms, winter metabolic suppression exceeds the passive effects of cooling. During dry seasons, estivating ectotherms can reduce metabolism without changes in body temperature, conserving energy reserves, and reducing gas exchange and its inevitable loss of water vapor. This overview explores the similarities and differences of metabolic suppression among these states within adult animals (excluding developmental diapause), and integrates levels of organization from the whole animal to the genome, where possible. Several similarities among these states are highlighted, including patterns and regulation of metabolic balance, fuel use, and mitochondrial metabolism. Differences among models are also apparent, particularly in whether the metabolic suppression is intrinsic to the tissue or depends on the whole-animal response. While in these hypometabolic states, tissues from many animals are tolerant of hypoxia/anoxia, ischemia/reperfusion, and disuse. These natural models may, therefore, serve as valuable and instructive models for biomedical research.
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Introduction

Metabolic energy is required for animal development, growth, maintenance, and reproduction, so the ability to obtain, store, and transform energy efficiently has come under intense selective pressure over evolutionary time. In many habitats, environmental conditions may constrain the ability of heterotrophic animals to obtain the food energy required to maintain rates of metabolism. For example, the lack of sunlight during polar winters brings primary productivity to a virtual halt, with knock-on effects for higher trophic level consumers. Under these conditions, sufficient food energy may still be available for very small mammals, for example, voles by foraging beneath the snow pack. Slightly larger mammals, such as ground squirrels and marmots, are too large for this subnivian niche, but too small to dig effectively through the snow, as do larger caribou and muskox. Even if energy can be obtained or stored, some environmental conditions may limit the rate at which this food energy can be transformed into usable ATP, or limit the total yield of ATP from that food. In deep, poorly mixed waters, where microbial decomposition outpaces photosynthesis, oxygen concentrations may be insufficient to support rates of aerobic metabolism. Some environmental conditions may constrain both energy availability and animals' abilities to maintain metabolic rates. Dry seasons typically not only have low productivity and, therefore, low energy availability, but may also constrain the ability of animals to metabolize energy. For air breathers, the gas exchange required to support aerobic metabolism is associated with some inevitable loss of water vapor from gas exchange surfaces. During dry seasons, therefore, the gas exchange required to maintain high rates of metabolism may result in unsustainable losses of body water.

Migration, among other potential advantages, allows many animals to spatially avoid challenging metabolic conditions. In the terrestrial realm, however, migration is generally limited to animals that can fly or are large enough to travel cross-country effectively and efficiently. Other animals adjust physiological systems so that rates of metabolism can be maintained despite environmental challenges. For example, when acclimated to hypoxic water, goldfish (*Carassius auratus*) remodel their gills, increasing surface area to maintain rates of gas exchange (198). Such adjustments have limits, however—large gill surface area will do little good if environmental oxygen is completely absent—and may have potentially negative physiological consequences. Indeed, the ability of goldfish to regulate blood ion composition may be compromised by the hypoxia-induced increase in gill surface area (198).

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An effective strategy for coping with energy stress is to temporarily avoid the environmental challenge by reducing the rate of metabolism until more favorable conditions return. Animals that undergo hibernation, torpor, and estivation exhibit amazing adaptations that give them the metabolic flexibility to survive in environments not available to most other animals. This metabolic flexibility is remarkably diverse across animal taxa, reflecting different evolutionary histories and environmental selective pressures. As a result, the patterns of reversible metabolic suppression are also quite diverse. Despite this diversity, research suggests many areas where mechanisms underlying reversible metabolic suppression converge, and I will highlight some of these mechanisms in this overview.

One of the most obvious and relevant differences among animal taxa is the pattern of thermoregulation (for a description of animal thermoregulatory patterns consult ref. 282). Since diverging from ectotherm ancestors, vertebrate endotherms have evolved a suite of behavioral, morphological, physiological, and biochemical traits that facilitate the generation of metabolically derived heat as well as mechanisms to regulate retention of that heat. These traits allow endotherms to regulate core body temperature (T_b) at fairly high and constant levels. As I discuss later, when endotherms enter hibernation or torpor this pattern of physiological thermoregulation is adjusted, but not abandoned. As a result, thermogenic metabolism can change rapidly during some stages of torpor bouts, and become temporarily decoupled from T_b. During these periods, Q_{10} values, traditionally used to relationship of metabolic rate (MR) with T_b, are of limited value. While reversible metabolic suppression is expressed in both endotherms and ectotherms, the metabolic response and its interaction with environmental temperature differs fundamentally between these two groups (Fig. 1), so I treat them separately in this overview.

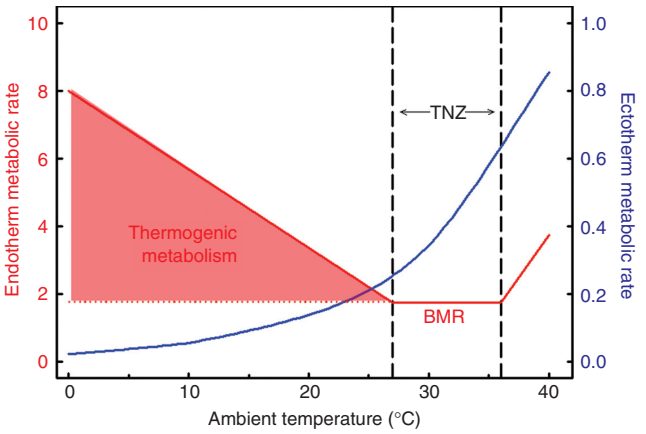

Figure 1 The effect of ambient (environmental) temperature on the metabolic rate of a typical nontorpid endotherm (e.g., a small eutherian mammal; red line) and an ectotherm vertebrate (blue line) of similar mass. Between 27 and 36°C (the Thermal Neutral Zone [TNZ], bounded by dashed lines) the endotherm metabolic rate is minimal, and T_b is maintained near 37°C by regulating heat loss, for example, through alterations in piloerection and peripheral blood flow. Below 27°C, the endotherm must increase thermogenesis, that is, increases in metabolism solely for the sake of producing more heat (e.g., shivering, activation of BAT) to defend T_b. The T_b of the ectotherm is very close to the ambient temperature and metabolism is regulated largely by passive thermal effects on enzyme-catalyzed reactions. Note the different scales on the two axes. For more details, consult ref. 282.

Metabolic Flexibility in Endotherms

Definitions and descriptions

For several decades, there has been a debate about the definitions and terminology used to describe metabolic flexibility in endotherms. The International Union of Physiological Sciences defines torpor as "A state of inactivity and reduced responsiveness to stimuli" (149). For several reasons (outlined in ref. 34), I believe that this definition is inadequate and even misleading, and I suspect that most people who have researched animals that undergo natural torpor would agree with my position. Resolving this debate is beyond the scope of this overview article, however, so instead of attempting to provide rigid definitions, I describe natural physiological states based largely on the change in MR, but also T_b.

This overview focuses on metabolic flexibility, so I use the word hibernation to describe a regulated state in which MR is reversibly suppressed to <10% of resting values for several days, with a corresponding decrease in T_b. Of course, there are exceptions to this description, especially in large mammals from the family Carnivora and animals that hibernate at very low ambient temperatures. For example, arctic ground squirrels hibernate in burrows above the frost line where an ambient temperature (T_a) of −16°C is not uncommon. During hibernation, these animals maintain a T_b near −2°C, but have metabolic rates that approach 50% of euthermic basal rates (45). The English word "hibernate" is derived from the Latin *hibernus*, which means "of winter," referring to the time of year in which hibernation usually occurs. This metabolic definition (<10% or resting MR) currently restricts hibernation to mammals but this is largely due to a lack of available data from birds. A recent review of heterothermy in free-ranging Caprimulgid birds demonstrates that the common poorwill (*Phalaenoptilus nuttalli*) may spend several days with T_b as low as 5°C (33). Data on the metabolic responses of these birds during these periods would be very valuable.

Daily torpor in endotherms is similar to hibernation, but the decrease in MR and T_b are generally less severe and last less than 24 h before being reversed. Most endotherms that exhibit daily torpor do so during cold seasons, but some display reversible metabolic suppression under hot and dry conditions, a state often referred to as estivation (from the Latin *aestas* meaning "summer"). Like other endotherms, these animals regulate T_b at fairly high and constant levels during the active season, but during inactive seasons, T_b fluctuates between high and low values over different time scales depending on the species. As such, they are often referred to as temporal heterotherms.

Endotherms in the cold

Endotherms are classically described as animals that use internally derived metabolic heat to maintain T_b a fairly high and constant levels. The evolution of fur, feathers, and subcutaneous fat helped vertebrate endotherms to reduce conductive and radiative heat loss to the environment. The insulating properties of pelage and plumage depend, in part, on thickness (distance between skin surface and bulk air/water) and density (number of underfur hairs/downy feathers cm^{-2}), both of which can change over evolutionary (e.g., among animals adapted to live at different latitudes and/or altitudes) and acclimatory time scales (e.g., seasonal shedding and molting). The thickness of this external insulation can also be altered acutely using piloerection. Heat retention can also be regulated acutely by physiological vasomotor control over conductive transfer of heat by blood from core organs toward the skin. Although these mechanisms of heat retention may be quite effective, it is also clear that endotherms have much higher rates of metabolism than ectotherms of similar size living at the same ambient temperature (note different axis scales on Fig. 1), so metabolic heat generation is clearly important to the endotherm strategy.

Most metabolic heat is derived from the mitochondrial electron transport system (ETS, as illustrated in Fig. 2). ETS complex I oxidizes NADH, produced mainly in the Krebs cycle, and sequential oxidation-reduction reactions by downstream ETS complexes release 56.2 kcal mol^{-1} of free energy (under standard conditions). In most mitochondria approximately 40% of this energy is used by ETS complexes I, III, and IV to pump protons from the mitochondrial matrix to the intermembrane space, establishing a proton motive force that can power ATP synthesis by ETS complex V. The rest of this free energy is released as heat. The high rate of metabolism in endotherms provides an endogenous heat source that can be used to regulate T_b.

Endotherms can maintain T_b using only basal metabolism (measured in resting, nonreproductive, postabsorptive, awake, calm, and nongrowing animals) within a narrow range of T_a termed the thermoneutral zone (TNZ, see Fig. 1). Within the TNZ, T_b is regulated by acutely adjusting the loss of metabolic heat through changes in piloerection, peripheral vasomotor tone, and evaporation (e.g., sweating, panting, and gular flutter), none of which incur significant metabolic costs. At the lower limit of the TNZ, these heat retention mechanisms are maximized, and the only way to maintain T_b is to produce more metabolic heat. The increase in metabolic rate above basal metabolic rate (BMR) is referred to as thermogenic metabolism. As illustrated in Figure 1, thermogenesis increases as T_a falls below the TNZ, and thermogenic metabolic demands for endotherms in the cold can be severalfold higher than BMR. Thermogenesis has been traditionally described as "shivering" or "nonshivering," but these designations tell us little about how heat generation is stimulated in a metabolic sense. Instead, I describe thermogenic metabolism that is either coupled to, or uncoupled from ATP synthesis. At T_a below the TNZ skeletal muscles of mammals and particularly birds shiver. This uncoordinated contraction of antagonistic muscle groups does no useful work, but results in high rates of ATP hydrolysis. This hydrolysis releases some heat itself (7.3 kcal mol^{-1}) but has its greatest thermogenic effect by stimulating flux through the ETS. The ADP released as a result of ATP hydrolysis binds to the central channel in ETS complex V (see Fig. 2), increasing the passage of protons from the intermembrane space into the matrix (211). This flow of protons not only powers ATP synthesis by complex V, thereby maintaining metabolic homeostasis, but also causes a temporary dissipation of the proton motive force. This dissipation stimulates ETS oxidation of reducing equivalents which reestablishes the proton motive force, but also releases considerable amounts of heat that can be used to regulate T_b (for an in-depth treatment of animal mitochondrial bioenergetics

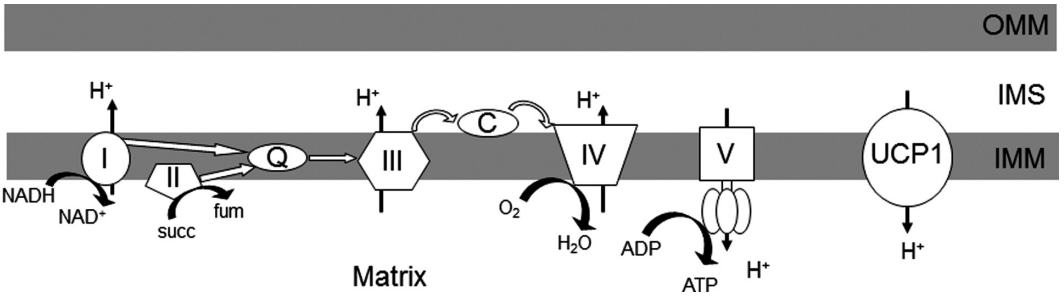

Figure 2 The ETS of a typical animal. The enzyme complexes are associated with the inner mitochondrial membrane (IMM). NADH, derived from the Krebs cycle, is oxidized by complex I and succinate by complex II. Electrons from these substrates are transferred to the mobile carrier coenzyme Q (Q), which 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. In the presence of ADP, derived from ATPase-catalyzed ATP hydrolysis, protons return to the matrix, powering ATP synthesis by complex V. The IMM of eutherian mammal BAT contain little complex V but, uniquely, significant amounts of UCP1. When BAT adipocytes are activated by sympathetic nervous stimulation, protons flow from the IMS to the matrix through UCP1, stimulating ETS substrate oxidation and heat production, but no ATP synthesis.

consult ref. 211). Heat produced by shivering, therefore, depends upon (is "coupled to") hydrolysis and resynthesis of ATP.

Mitochondria in specialized tissues of some eutherian mammals contribute to thermogenic metabolism that is not coupled to the synthesis and hydrolysis of ATP. The mitochondria of brown adipose tissue (BAT) express very little ETS complex V, but a great deal of uncoupling protein 1 (UCP1; Fig. 2). When BAT cells are activated by sympathetic stimulation, UCP1 translocates protons from the mitochondrial intermembrane space to the matrix. Though the mechanism underlying this translocation remains a matter of debate (79), as in any mitochondria, this dissipation of the proton motive force stimulates flux through the ETS, thereby releasing more heat with virtually no ATP synthesis (162). Since 1997, there has been considerable research interest in mitochondrial proteins with varying degrees of similarity to UCP1. These UCP "analogs" (especially UCP2 and UCP3) do uncouple mitochondrial substrate oxidation from ADP phosphorylation under specific conditions and this function may have important physiological consequences, for instance, in preventing oxidative damage, but they do not appear to contribute significantly to thermogenesis (79).

In temperate, subpolar and polar regions, winter poses a great challenge to endotherms. Low T_a increases the demand for thermogenic metabolism, but food availability is typically at a nadir. Without adequate food energy endotherms may not be able to fuel thermogenesis and may enter hypothermia, an uncontrolled decrease in T_b that can cause death if not reversed by the application of exogenous heat. Despite these challenges, many endotherms inhabit these areas and remain euthermic throughout the winter. They are able to do so in part by maximizing mechanisms of heat retention, but they still need a supply of food energy. Continued foraging throughout the winter can supply this energy, but many animals also use energy stored within their bodies as adipose tissue (1), an approach that is better suited to large animals with their greater allometric capacity for storage. Many smaller endotherms use food stored during warmer months to power thermogenic metabolism through the winter. For example, American red squirrels (*Tamiascurius hudsonicus*) cache spruce cones and feed on the seeds throughout winter. Sufficient food stores allow red squirrels to remain euthermic throughout the winter, but survival depends on MR not outpacing food stores (170).

Many endotherms, use a different strategy to deal with the energetic challenges of winter. These animals decrease the demand for metabolic energy, prolonging their ability to survive in environments with low energy availability. Many of these animals, especially small species, are able to withstand low T_b, and adjust the T_b they defend to much lower levels during most of these cold periods. This strategy reduces the lower limit of the TNZ, thereby reducing thermogenic energy demands (see Fig. 1). The reduction in T_b also passively reduces tissue metabolism by Arrhenius effects. On the other hand, most temporal endotherms also invoke mechanisms of active, T_b-independent metabolic suppression that permit further energy savings.

The physiological adjustments made by temporal heterotherms are often assumed to be adaptations selected for surviving in challenging environments. It is evident, however, that some species that employ hibernation live in very similar environments as closely related species that remain euthermic throughout the winter. For example, the red squirrel and the Arctic ground squirrel (*Urocitellus parryii*) are both successful in overlapping subpolar regions with long and cold winters. While the red squirrel remains euthermic (32), the Arctic ground squirrel, a member of the same mammalian Rodent family (Sciuridae) spends up to 9 months hibernating, allowing T_b to fall as low as −1.9°C (18).

Animals that undergo hibernation and daily torpor possess several physiological characteristics that distinguish them from typical endotherms. In this overview article, I review some of these distinctions and the mechanisms underlying them, including the profound readjustment of thermoregulation and the ability to suppress metabolic rate. Beyond conserving metabolic energy over a cold winter, these physiological characteristics have consequences for other environmental challenges and potential applications to pathological conditions that I will also explore.

Temporal heterothermy: Relationships and patterns

Hibernation and torpor occur in species representing at least 10 mammalian orders (116; Fig. 3) and 12 avian families (192; Fig. 4). A recent meta-analysis presents a thorough phylogenetic analysis of heterotherms, and suggests that daily torpor and hibernation are two distinct phenotypes rather than ends of a continuum (245). The divergent winter energetic strategies within a single mammalian family, exemplified by the red squirrel and Arctic ground squirrel mentioned earlier, raises questions about whether temporal heterothermy is ancestral or derived. The so-called ground squirrels include many species known to hibernate. Recent molecular data suggest that the Marmotini, the rodent tribe that includes the ground squirrels, diverged relatively recently from other Sciurid groups (251), suggesting that hibernation may be a relatively recent evolutionary occurrence. Obligate hibernation is even expressed in at least one strepsirrhine primate, *Cheirogaleus medius* (72). Explaining the evolutionary origins of heterothermy is beyond the scope this overview, so I refer the interested reader to these excellent reviews (179, 180, 245). From a physiological and metabolic perspective, however, I describe different patterns within temporal heterotherms and the mechanisms that might contribute to their expression.

Obligate versus facultative hibernators

Hibernation appears to be restricted to mammals (with the possible exception of the poorwill, a Caprimulgid bird). Obligate hibernators follow an endogenous circannual rhythm and hibernate each year, regardless of environmental

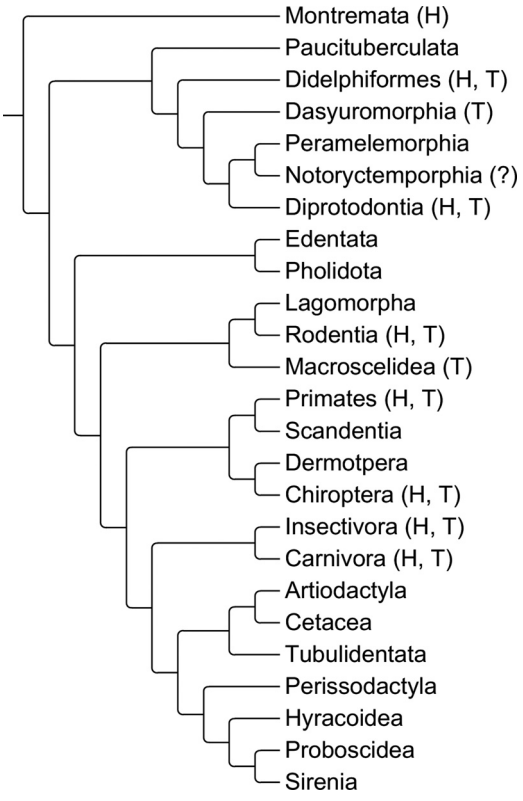

Figure 3 Phylogenetic tree showing mammalian orders with species that use hibernation (H) or torpor (T). Modified with permission from ref. 115.

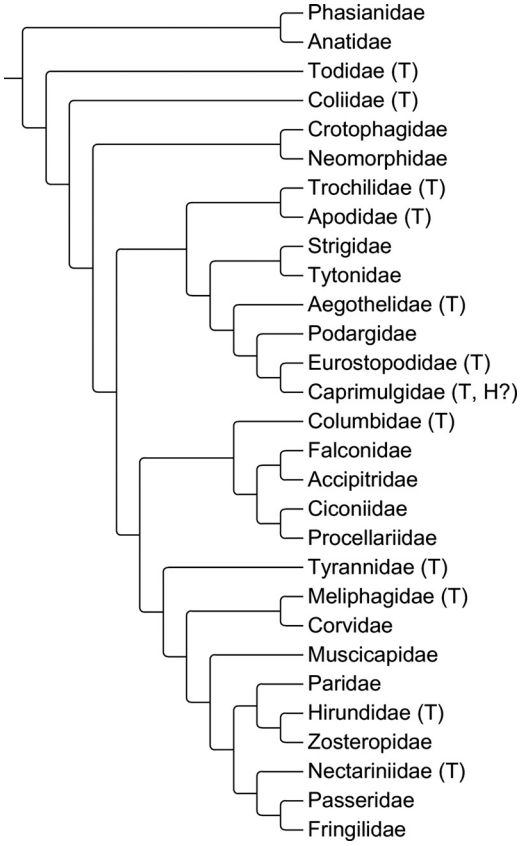

Figure 4 Avian phylogenetic tree showing families with species where adults display torpor (T) or possible hibernation (H?). Modified with permission from ref. 192.

conditions. This fact is dramatically illustrated by the research of Pengelley and colleagues, who 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, at least in the first year following birth (225, 226). It is common to see late-summer or autumn torpor in captive ground squirrels housed at warm ambient temperatures and long photoperiods after they have reached their peak body mass or adiposity (247). In the summer, obligate hibernators do not hibernate, even when exposed to winter-like light and temperature. In most obligate hibernators, adult males begin hibernation earlier in the autumn, and terminate hibernation earlier in the spring than females (e.g., ref. 17). Mechanisms underlying this endogenous rhythm are largely unknown, but the pattern is reflected by proteomic studies that, in general, show greater differences among seasons than among hibernation phases within the winter. One significant example is represented by the changes in the BAT in a hibernator. In most small mammals, proliferation of BAT requires several weeks of acclimation to low T_a and/or short photoperiod. In thirteen lined grounds squirrels (*Ictidomys tridecemlineatus*), however, several metrics of BAT proliferation precede the environmental transition from summer to winter (141).

The free-running cycle of this endogenous rhythm has a period of approximately 300 days in golden-mantled ground squirrels (225, 226). The cycle is entrained and affected by environmental conditions (see ref. 305 for an excellent review), as illustrated by comparisons of three woodchuck (*Marmota monax*) populations spread over a large latitudinal gradient. Not surprisingly, in the wild the more polar (northerly) population starts hibernating earlier in the autumn, stops hibernating later in the spring and spends more total time torpid compared with more southern populations (314). When maintained in the laboratory under common conditions, however, the woodchucks expressed many hibernation characteristics similar to the wild population from which they originated (95, 314). This result suggests that some components of the endogenous rhythm are fixed, and that the populations have diverged genetically, so it would be interesting to compare gene expression patterns and corresponding hibernation characteristics of these populations and, perhaps, offspring of northern and southern parents.

This endogenous circannual cycle of hibernation expression is also reflected in food intake and body mass in marmots (302) and ground squirrels (8) maintained under constant laboratory conditions. These cycles are also evident in free living adult Arctic ground squirrels, where the increase in body mass seen prior to hibernation is due almost entirely to a seven-fold to eight-fold increase in body fat and can result

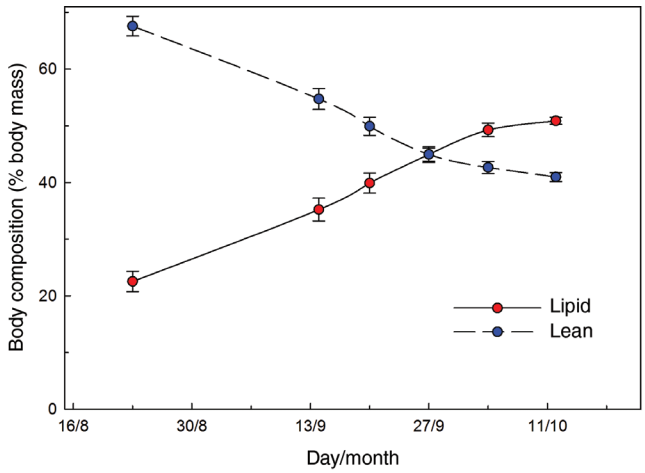

Figure 5 Body composition of young-of-the-year thirteen-lined ground squirrels. Shortly after weaning animals were weighed and body composition was determined using quantitative H^+ nuclear magnetic resonance. See ref. 125 for experimental details. Values are means ± standard error of three males and four females from the same litter. J.F. Staples, D.J. Chung, and C.G. Guglielmo, unpublished data.

in >50% of female body mass consisting of lipid (255). As in other mammals this increase in body fat corresponds with an increase in circulating leptin in some hibernators (98), but in others there may be a dissociation between lipid mass and leptin secretion (reviewed in ref. 97). Perhaps more impressive is the weight gain achieved by young-of-the-year obligate hibernators. Female thirteen lined ground squirrels in southern Manitoba deliver litters in late May or early June with weaning occurring approximately 30 days after parturition. These young-of-the-year must continue to grow and accumulate sufficient endogenous energy stores to survive hibernation throughout the approaching winter. In captive-bred animals, we found that recently weaned pups weigh approximately 110 g and are fairly lean, with 25% of body mass consisting of lipid. As illustrated in Figure 5, over the subsequent summer and early autumn weeks lean mass changes little, but body mass approximately doubles before peaking in autumn. Virtually all of this mass gain is attributable to increases in body lipid. This dramatic annual cycle in hibernators promises to inform us about the regulation of food intake and lipid storage (reviewed in ref. 97; see *Fuelling Temporal Heterothermy*, later).

The pattern of obligate hibernation can also be affected by local environmental conditions. For example, Eastern chipmunks (*Tamias striatus*) are obligate hibernators that rely on cached food to supply metabolic energy throughout the winter, rather than endogenous lipid stores. Supplementing natural food availability decreases the proportion of time these animals spend torpid, and torpor depth (minimal torpid T_b, as suggested by skin temperature; ref. 147). Alterations of specific dietary nutrients, especially polyunsaturated fatty acids (PUFA), can also alter torpor patterns (see *Fuelling Temporal Heterothermy*, later). Supplementing diet with items that mimic the PUFA profile of natural forage (i.e., changing food

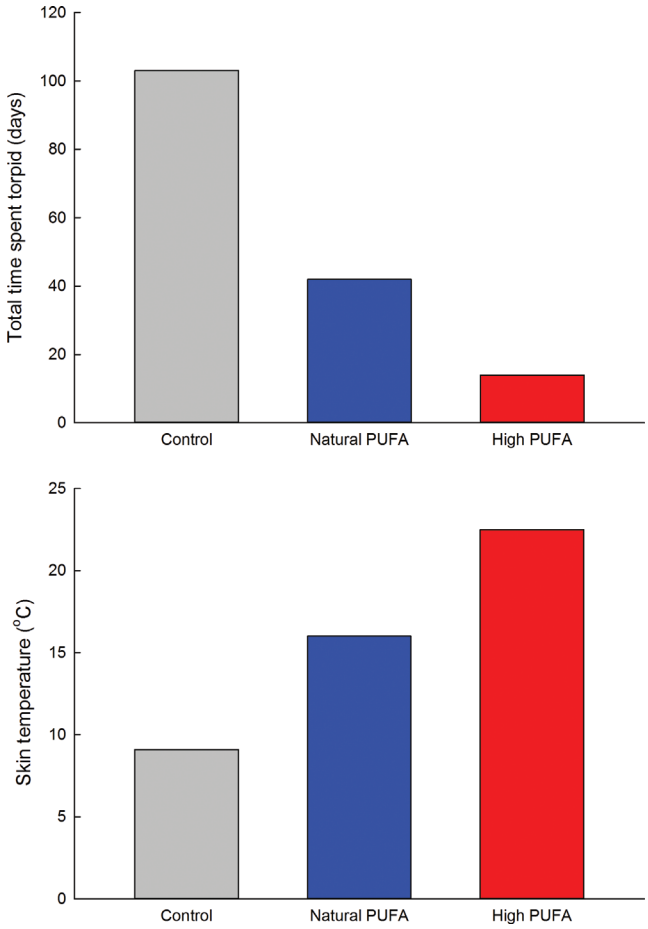

Figure 6 The effect of food quantity and PUFA content on hibernation in eastern chipmunks (*Tamias striatus*). Animals were fed either on natural forage (Control) or had their diet supplemented with food that had a similar PUFA content to the natural diet (Natural PUFA) or with food containing higher PUFA than the natural diet (High PUFA). Data from ref. 201 with permission.

quantity, but not dietary PUFA quality) decreases the time spent torpid and the torpor depth (201). As seen in Figure 6, increasing the amount of PUFA in food supplements enhances this effect of food quantity (201).

In contrast to obligate hibernation, the expression and characteristics of hibernation in some mammals can differ greatly within a species, and depend largely on environmental conditions. The term "facultative" is frequently used to describe the hibernation pattern in these animals. Syrian hamsters (*Mesocricetus auratus*) do not appear to have a free-running endogenous hibernation rhythm and can be induced to hibernate at any time of the year, but only after acclimation to short photoperiod and low T_a (281). In the wild, hibernation expression in black-footed prairie dogs (*Cynomys ludovicianus*) has been reported to range from absent (14) to typical of obligate hibernators (175), even when sampled from similar geographic areas. Local environmental conditions, such as T_a and rainfall, likely influence heterothermy patterns in these facultative hibernators.

Daily torpor: Seasonal versus fasting induced

Compared with hibernation, daily torpor is used to describe periods during which endotherms reduce MR to levels not below about 26% of BMR and T_b to levels typically not below 11°C (245). These periods last less than 24 h and occur during the animal's inactive period (116). This pattern of metabolic flexibility offers significant energy savings while still permitting foraging during the active period of the circadian cycle.

In some daily heterotherms, the expression of torpor requires a fairly long acclimation to short photoperiods and/or low T_a. This seasonal daily torpor occurs even when food is abundant, though diet quantity and quality can affect torpor patterns (246). Expression of daily torpor can also depend on social and, likely, thermal interactions—virtually all solitary striped skunks (*Mephitis mephitis*) express daily torpor (as described by drops in T_b), while torpor was observed only rarely in skunks that pass the winter in communal dens (148).

In other endotherms, such as the house mouse (*Mus musculus*) torpor can be induced by withholding food even when animals are housed at a fairly high (~20°C) T_a with a 12 h photoperiod (42). This fasting-induced torpor stops when food is reintroduced (see Fig. 7 for example). Recently, a training protocol has been devised to manipulate fasting-induced torpor by requiring mice to perform exercise to obtain food (276). By increasing the workload required to obtain a food reward, mice lose body mass and enter "workload" torpor in the light phase of the photoperiod. This ingenious method holds promise for investigating the interaction between torpor expression and energy balance at several different levels of organization. It has been reported anecdotally that fasting-induced torpor in mice can be mimicked by administration of 2-deoxy-glucose (112), a glucose analogue that inhibits glycolysis. In Djungarian hamsters (*Phodopus sungorus*), however, 2-deoxglucose and mercaptoacetate (which inhibits fatty acid oxidation) actually decrease torpor expression (264). I suspect that, like adenosine 5′-monophosphate (see *How is Torpor Induced?*, later), 2-deoxy-glucose is one of many substances that can induce temporary hypothermia, rather than torpor.

Estivation

Estivation in endotherms has been used to describe reversible metabolic suppression with altered thermoregulation at high ambient temperatures. Estivation is sometimes difficult to recognize based on measurements of T_b because the high summer T_a results passively in high T_b (55). Moreover it can be difficult to distinguish between estivation and hibernation, as some animals become torpid at the end of summer, and remain metabolically suppressed throughout autumn and winter (reviewed in ref. 117).

Several bird species enter torpor during the summer (reviewed in ref. 117). In mammals, estivation is expressed in monotremes (e.g., echidna, *Tachyglossus aculeatus*) marsupials (e.g., dunnarts, *Sminthopsis spp.*) and placentals,

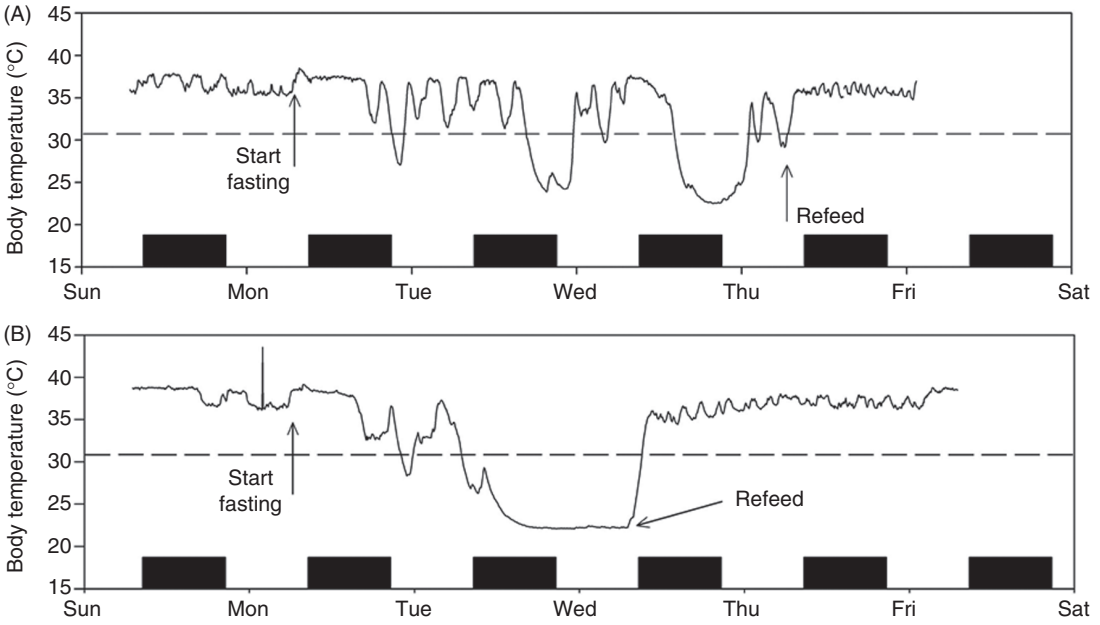

Figure 7 Fasting induced daily torpor in mice (*Mus musculus*). Body temperature T_b of two individual Balb/c mice over the course of a week during which fasting occurred. Photophase began at 9 pm, and scotophase (black bars) began at 9 am. Fasting began just prior to the start of the scotophase on Monday (7 am) and ended 3 days later. Prior to fasting, mice maintained a fairly constant T_b and torpor was not observed. Once fasting began, T_b became more variable, and bouts of daily torpor occurred, where T_b dropped below 31°C (indicated by dashed line). These mice underwent one or more bouts of daily torpor on each of the 3 days of fasting. Once food was returned, T_b stabilized near 37°C, and no bouts of torpor were observed. Modified from ref. 42 with permission.

especially bats. In recent years, the use of torpor by tropical and subtropical mammals, including primates, has received increasing attention (73). While this metabolic suppression certainly reduces energy expenditure, the associated decrease in gas exchange also reduces evaporative water loss (117), probably a key selective pressure during the summer in these environments. It appears, however, that the physiological patterns and underlying mechanisms of metabolic suppression under hot and dry conditions are not distinguishable from other forms of temporal heterothermy, and the term "summer torpor" has been used more frequently in recent years (73, 117). In my opinion, the term estivation is best reserved for ectotherms (see *Metabolic Flexibility in Ectotherms*, later).

Whether hibernation and daily torpor are best considered as extreme points on a continuum (55) or separate and distinct phenotypes (245) is a matter of active debate. A few species, including some bats (155) and the edible dormouse, *Glis glis*, (which also uses torpor in the summer; 307) employ both daily torpor and hibernation. The terminology used to describe temporal heterothermy may also add to confusion in the uninitiated reader—hibernators experience several episodes of reduced MR and T_b throughout the hibernation season, and each episode is referred to as a torpor bout.

Patterns of metabolic suppression

Heterothermy results in energy savings that arise from a combination of three effects: (i) adjustments in thermoregulation that remove stimulation from thermogenic tissues such as skeletal muscle and BAT, thereby reducing thermogenic metabolism, (ii) passive thermal effects due to reduced T_b (the so-called Q_{10}, or Arrhenius effect), and (iii) active, temperature-independent metabolic suppression below basal levels in nonthermogenic tissues. All three of these components can contribute to the reduction of MR in heterotherms, but the contribution of each component depends, to some extent, on body mass, the pattern of heterothermy, and the environmental conditions.

In the autumn, obligate hibernators inhabiting temperate, subpolar and polar regions begin hibernation, but the hibernation season is not one uninterrupted period of reduced MR and T_b. Instead hibernators undergo a series of discrete torpor bouts that are separated by periods during which T_b returns to levels near 37°C (see Fig. 8 for examples from ground squirrels). Each of these bouts can be divided into four discrete stages: (i) entrance, where MR falls rapidly by over 90%, and T_b falls subsequently toward T_a, (ii) torpor, where MR and T_b remain low and fairly constant for several days, (iii) arousal, where MR spontaneously increases rapidly over a few hours, followed by a rise in T_b to ca. 37°C, and (iv) interbout euthermia (IBE; sometimes also referred to as interbout arousal), where MR and T_b remain high and fairly constant for several hours before a new torpor bout begins (Fig. 8B). The reduction in MR in entrance and torpor undoubtedly conserves

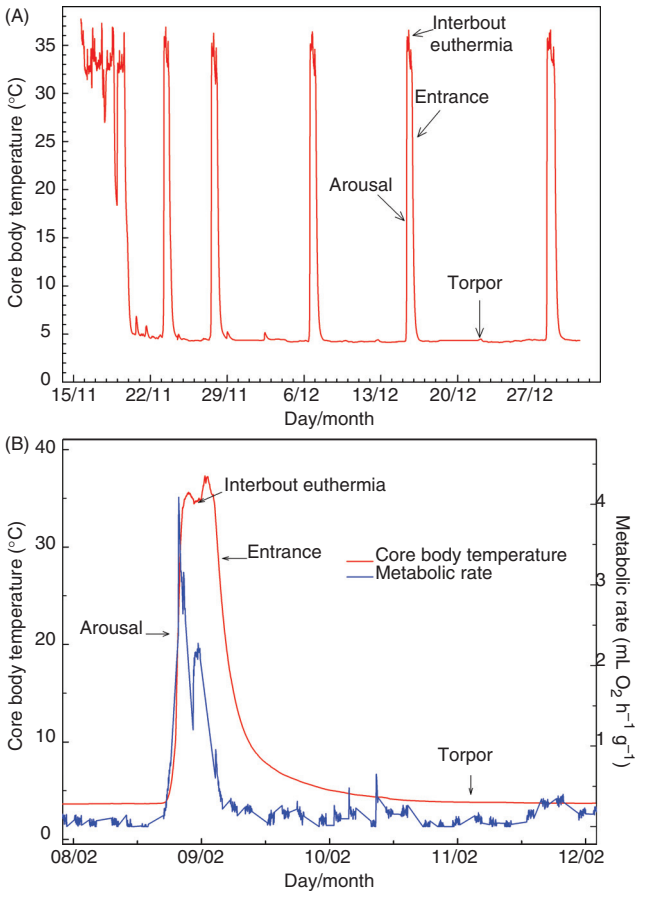

Figure 8 Core body temperature of a thirteen lined ground squirrel through several torpor bouts at the beginning of the hibernation season (A). Metabolic rate and body temperature of a ground squirrel in the different stages of a torpor bout (B). Modified from ref. 266 with permission.

energy, but arousal and IBE are quite expensive. The increase in T_b can be realized entirely from endogenously produced metabolic heat, distinguishing hibernation from hypothermia; the T_b of hypothermic endotherms can only be increased by application of exogenous heat. Exogenous sources of heat, for example from conspecifics sharing the hibernaculum (12) can also induce and accelerate arousals. Nonetheless, 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 periods (298), though the cost of these arousals depends on the conditions under which hibernation occurs (152).

Similar to hibernation, daily torpor is initiated by a decrease in MR, followed by a drop in T_b (Fig. 9), but the decreases are less severe and a torpor bout lasts only a few hours before it spontaneously ends. The energy savings are less substantial than hibernation but arousing daily permits foraging throughout the winter. Unlike hibernators, daily heterotherms may rely more on passive thermal effects to reduce MR as T_b falls.

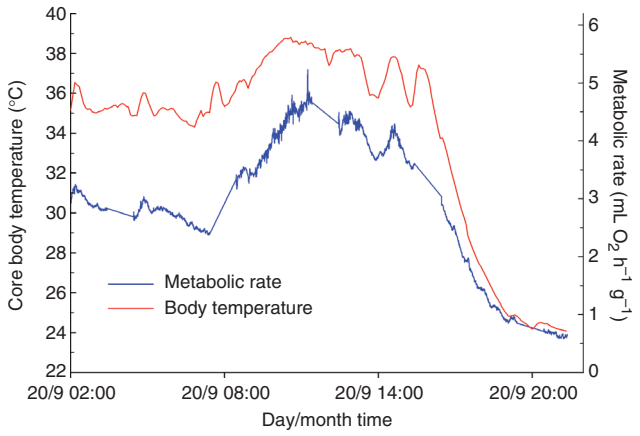

Figure 9 Body temperature and metabolic rate of a Djungarian hamster showing a spontaneous bout of daily torpor. Modified from ref. 134 with permission.

Adjusting thermoregulation

In endotherms, T_b is regulated within a narrow range referred to as the thermoregulatory or body temperature set point (T_{set}). In mammals, T_{set} is determined largely by neurons within the preoptic region of the hypothalamus (27). As T_a declines, conductive and radiative heat loss increases leading to small decreases of T_b. If T_b falls below T_{set} a series of heat conservation mechanisms are initiated (see ref. 282 for review) and, within the TNZ, these adjustments are sufficient to defend T_b close to T_{set}. At T_a below the TNZ, however, endotherms must initiate thermogenic metabolism to defend T_b.

In mammals and birds, acute exposures to T_a below the TNZ invoke ATP-coupled thermogenesis in the form of shivering. As mammals acclimate to cold exposure, however, shivering thermogenesis declines as BAT-mediated uncoupled thermogenesis increases (75). During cold acclimation and in preparation for hibernation, ground squirrels increase BAT mitochondrial abundance (195), and expression of several genes including those coding for UCP1 (126) thereby increasing nonshivering thermogenic capacity. Even before hibernation begins, however, evidence suggests that Arctic ground squirrels adjust thermoregulation to defend a T_b slightly, but significantly, below the typical summer levels that are near 37°C (254).

During entrance into a torpor bout, the lower limit of T_{set} progressively decreases (136). Although the precise mechanism regulating this T_{set} decrease remains unknown, it may relate to increased sensitivity of central nervous system purinergic signaling mediated by adenosine A(1) receptors (153). The decrease in T_{set} represents a resetting, as opposed to an abandonment, of thermoregulation. At T_a above 0°C Arctic ground squirrels in the torpid phase of hibernation will thermoconform, and MR does not change appreciably until T_a increases above 16°C (47). If T_a falls below 0°C, however, T_b is defended around −0.4°C by an increase in thermogenic metabolism (47).

During entrance into torpor, the decrease in T_{set} will effectively shift the TNZ to lower T_a (see Fig. 1). For most animals that hibernate below the frost line, the T_a within their burrows will be above the lower limit of the TNZ, so that thermogenesis will cease during entrance. As a result, MR will drop and, in all but the smallest mammals, this decrease in metabolism will occur even before T_b starts to fall. An example can be seen in Figure 8B where, during entrance, ground squirrel MR declines precipitously with no drop in T_b, likely reflecting the resetting of T_{set} and cessation of BAT-mediated thermogenesis. During arousal the lower limit of the T_{set} increases toward euthermic levels, so that T_a is suddenly well below the lower level of the TNZ. As a result, thermogenenesis is activated, resulting in a large increase MR before T_b changes. At least in small eutherian heterotherms, this initial burst in thermogenesis is believed to be BAT-mediated; however, understanding the function of BAT at such low T_b has not be studied thoroughly.

In small heterotherms, thermogenic metabolism during euthermic periods at cold T_a can be several-fold higher than BMR, so this adjustment of thermoregulation during entrance likely accounts for the greatest proportion of energy savings in a torpor bout. For example, in Figure 8B the decline in ground squirrel MR during entrance is substantial before T_b begins to decline. Of course, it is also possible that temperature-independent metabolic suppression occurs simultaneously in nonthermogenic tissues, accentuating the decline in MR. The resetting of T_{set}, however, inevitably leads to a decrease in T_b, resulting in decreases in metabolic rate through passive thermal effects.

Body temperature effects

Passive thermal effects due to reduced T_b can certainly contribute to energy savings in both hibernation and daily torpor. A decrease in T_b will slow the rates of most enzyme catalyzed reactions by effects on protein structure and stability, enzyme-substrate binding, and enzyme turnover number (reviewed in ref. 282). The contribution of passive thermal effects to metabolic savings in heterotherms remains unclear, however, for several reasons. Firstly, as mentioned in the previous section in both hibernation (Fig. 8A) and daily torpor (Fig. 9) MR declines before T_b begins to fall. Secondly, in some hibernators, changes in T_b during the torpid phase do not affect MR (47). Thirdly, as mentioned in the next section, black bears achieve considerable metabolic suppression with little change in T_b. Finally, artificial cooling of nonhibernating mammals yields much higher mass-specific MRs than hibernation. In the summer, ground squirrels do not (cannot?) hibernate. It is, however, possible to reduce the T_b of summer ground squirrels to levels comparable to that of hibernating conspecifics using mild anesthesia and external cooling. By using this induced hypothermia, Wang et al. (297) found that simply cooling the body of a ground squirrel could not account for the decrease in MR observed in hibernation. In fact, the MR of the hypothermic summer animals were approximately

15-fold higher than hibernators at the same T_b (297). Clearly, the drop in T_b alone cannot account for all of the metabolic suppression seen in hibernation and daily torpor.

Metabolic heat is produced by tissues within the volume of an animal, but is exchanged with environment over its surface. Because the relationship between surface area and volume changes with body mass, the relative contributions of thermogenic adjustments and passive thermal effects to the metabolic savings available to heterotherms depends, in part, on body size.

Body mass effects

Body mass affects which animals use temporal heterothermy and the pattern of heterothermy expression. It has been claimed that no animals larger than approximately 10 kg hibernate, a claim that may seem reasonable if T_b is used as the only criteria. The advantages of hibernation may be limited for large animals because their low rates of heat loss mean that they do not rely as heavily on thermogenesis to maintain euthermic T_b. Moreover, even very low metabolic rates produce sufficient heat to prevent them from attaining very low T_b seen in small hibernators (see later for details), so MR reduction due to passive cooling would be minimal. On the other hand, maintaining fairly high T_b while hibernating may actually be advantageous.

The basal and maximal MRs of animals scale allometrically with body mass so that large animals have disproportionately low mass-specific MRs (116) and broader TNZs (240). In contrast to this pattern, the mass-specific MR of animals hibernating at low T_b appears to be less dependent on body mass, that is to say MR in the torpid phase scales with a lower allometric exponent than basal MR (116). Taken together, these observations have been interpreted to mean that the minimum metabolic requirement to maintain cellular viability is common to all mammals, and that hibernation reduces metabolism toward this level (135). In fact, the estimated basal, mass-specific MR of the blue whale converges on the lowest MRs recorded from mammalian hibernators (257). For small mammals that have high mass-specific MRs and require considerable thermogenesis to defend euthermic T_b, the energy savings offered by hibernation are considerable. From an energetic standpoint, however, hibernation probably offered less of a selective advantage for larger mammals over evolutionary time.

In small hibernators, energetic savings are realized by reducing thermogenic metabolism, passive cooling, and active metabolic suppression. For large animals, however, the ability to decrease thermogenic metabolism and passively cool is likely limited. Heat produced by oxidative metabolism (see *Endotherms in the Cold*, earlier) within body tissues is lost to the environment by radiation and conduction (facilitated by convection) across body surfaces, at least when T_b is greater than T_a. The T_b depends on the balance between the rate of metabolic heat production and these conductive and radiative heat losses. This balance depends, in part, on the ratio of body surface area (over which heat is lost) and body volume (representing the amount of tissue producing heat). The lower surface area/volume ratio of larger animals means that, for a given mass-specific MR, relatively less metabolic heat can be lost to the environment, resulting in a higher T_b. Recently, this constraint on heat dissipation has been considered a selective pressure that has shaped the evolution of life history patterns within mammals (259).

With regard to metabolic suppression in heterothermy, the relatively low surface area/volume ratio of large mammals may constrain their ability to reduce T_b even if MR is actively suppressed. Hochachka and Guppy (144) calculated that if a 100 kg bear, housed at a T_a of 5°C, produced metabolic heat at the same mass-specific rate as a hibernating 400 g ground squirrel (with T_b of 5°C), the T_b of that bear would be no lower than 33°C. This modeling exercise assumes typical fur insulation on an animal in the "balled up" posture normal for hibernating bears. While interesting, how does this model compare with measurements from real animals?

From autumn to spring, black bears (*Ursus americanus*) and grizzly bears (*U. arctos*) retreat to dens, become inactive and neither eat, drink, urinate nor defecate. During this "winter lethargy" MR may be reduced to 25% of BMR (284). While this degree of metabolic suppression may appear modest compared with the "deep" hibernation typified by ground squirrels, the actual mass-specific MR is close to the lowest MR observed in any mammal (247). Throughout this period, bear T_b remains between 30 and 36°C. These real data compare favorably with the modeling exercise mentioned in the previous paragraph. The impressive degree of metabolic suppression, in the absence of any profound cooling, leads me to consider the bear to be a legitimate hibernator, at least from a metabolic perspective.

In effect, the surface area/volume of the bear is so small that it cannot cool below about 30°C even after MR has been suppressed to minimal levels. If the bear was able to reduce T_b to 5°C, typical of many small hibernators, passive thermal effects would lower mass-specific MR to levels close to that of a 250 g thirteen lined ground squirrel (Fig. 8; assuming a Q_{10} of 2.5). Bears likely have the capacity to reduce T_b during hibernation by shedding insulating fur, maximally vasodilating peripheral blood vessels and adopting a sprawled posture to maximize surface area for conductive and radiative heat loss. The fact that they do none of these things may relate to the influence of selective pressures other than energy savings on hibernation patterns. Hibernation at a low T_b may have drawbacks. In animals that hibernate at low T_b (i.e., near 5°C), the duration of torpor phases depends on body mass within (313) and among species (104). In woodchucks (313) and edible dormice (21), larger, fatter animals spend less time torpid, and have higher torpid T_b. These results are at least analogous to the reduced frequency and depth of torpor in hibernating chipmunks with large, supplemented food caches (Fig. 6; ref. 147) and suggest that, when energy supplies permit, animals minimize torpor bout duration and depth,

presumably to minimize potential negative consequences of low T_b on, for example, immune system function (248).

In the two preceding sections, I outlined how heterotherms may reduce metabolic expenditures by adjusting thermoregulation to reduce thermogenic metabolism, and allowing T_b to fall to reduce tissue metabolism through passive thermal effects. Combined, are these two effects sufficient to explain the suppression of metabolism seen in hibernation and daily torpor? A comparative meta-analysis suggests that decreasing T_{set} and allowing T_b to fall may explain the suppression of metabolism in daily torpor (116). However recent studies demonstrate significant temperature-independent suppression of mitochondrial oxidative metabolism in daily torpor (40, 167). Moreover, there are significant reductions in MR during fasting-induced daily torpor in golden spiny mice (*Acomys russatus*) at thermoneutral temperatures with virtually no change in T_b (123), a similar result to earlier studies on the edible dormouse, *Glis glis* (90). Under these conditions, thermogenic metabolism should be minimal, but during entrance, MR declines below basal levels even before T_b falls. These results suggest that both hibernation and daily torpor involve regulated, temperature-independent suppression of metabolism in nonthermogenic tissues. In the following sections, I explore aspects of this "active" metabolic suppression, but it is clear that there is a requirement for more data to better quantify the relative contribution of each of these components to MR reduction in heterothermy.

Regulating metabolism in hibernation and daily torpor

In temporal heterotherms, whole-animal MR, usually measured as oxygen consumption, may vary by as much as 100-fold between torpor and arousal. While some of these changes are due to thermoregulatory adjustments and passive thermal effects, much of it may be due to active, regulated suppression of metabolism (133). Classically, regulation of oxidative metabolism has been regarded as a feedback between demand and supply; for example, as demand for ATP increases during muscular work, the increased supply of ADP to mitochondria stimulates ATP production and oxidative metabolism (e.g., ref. 57), though opinions differ, depending on the tissue (15). ATP and its hydrolysis products can act as powerful modulators of enzyme activity (229), so any imbalance between ATP supply and demand between torpor and arousal would have significant metabolic consequences for temporal heterotherms. I would predict, therefore, that both ATP supply and demand are altered in synchrony throughout the reversible metabolic suppression displayed by temporal heterotherms.

Does energy metabolism remain balanced in temporal heterothermy?

Direct measurements of tissue high-energy phosphates in temporal heterotherms are rare, especially for daily torpor. Moreover, some of these experiments compared animals that were not hibernating (summer or early autumn) with winter animals that had been hibernating for several weeks. Many aspects of physiology, molecular biology, and cellular metabolism change with season, independent of heterothermy (188). While these seasonal differences are inherently interesting, to understand the changes associated with reversible metabolic suppression *per se* it is best to compare winter animals that are in steady-state torpor with other winter animals in IBE, arousal and/or entrance.

Despite the limitations of the existing data, some useful information does emerge. In skeletal muscle from jumping mice (*Zapus hudsonius*; ref. 274), golden-mantled ground squirrels (183) and white-tailed prairie dogs (*Cynomus leucurus*; ref. 91), ATP concentrations may decline by more than 50% in hibernation, but in some cases, such declines are not matched by corresponding increases of ADP or AMP (183, 274). Instead, there is a decrease in total tissue adenylate concentrations, presumably by deamination of adenosine. As a result, adenylate energy charge and AMP, thought to be important in regulation of many metabolic enzymes and signaling pathways including AMP activated protein kinase (AMPK), remain unchanged between torpor and euthermia. In contrast to this pattern, skeletal muscle ATP content has been reported to remain constant between euthermia and hibernation in jerboas (*Jaculus orientalis*), though AMP content decreases by about 50% (87).

In the brain of 13-lined grounds squirrels, ^{1}H nuclear magnetic resonance (NMR) spectroscopy showed that creatine phosphate increased approximately 33% from euthermia in the autumn to torpor in the winter (137). While this is an interesting finding on its own, it is more informative to note that concentrations of this high-energy phosphate did not differ between the low MR state of torpor to the high MR state of IBE (137). These data suggest that oxidative metabolism remains well balanced throughout the hibernation cycle, at least when steady states are compared. This carefully controlled study illustrates the importance of using appropriate comparison groups in hibernation studies.

The data available for liver also suggest that metabolism remains balanced throughout the different phases of hibernation. Using traditional "wet" biochemistry we found that, in golden-mantled ground squirrels, whole liver ATP and ADP content does not change among summer euthermia, winter torpor and IBE (269). Perhaps more interesting than these static measurements is monitoring ATP throughout the dynamic transition between torpor and euthermia. Using ^{31}P nuclear magnetic resonance spectroscopy we demonstrated that liver ATP does not change significantly during 60 minutes of arousal (268). Unfortunately, these data were collected only during induced arousals, when the animals were removed from the cold, dark environment chamber to a bright, warm NMR room; we have not yet found a feasible way to induce torpor within the bore of a superconducting magnetic to monitor high energy phosphates throughout entrance or spontaneous arousal.

Overall, the available data suggest that, despite potential differences among tissue types and, perhaps, species, ATP supply, and demand remain largely balanced despite the enormous changes in whole-animal MR throughout the hibernation cycle. Retaining this balance would require coordinated suppression of pathways that consume and produce ATP.

Turning down ATP consumption

Rolf and Brand (241) estimated that, in mammals, cellular ATP demand is accounted for by the following processes: ion pumping by Na^+/K^+ ATPase and Ca^{2+} ATPase (accounting for up to 38% of total O_2 consumption), protein synthesis (up to 28%), gluconeogenesis (10%), actinomyosin ATPase (10%), and ureagenesis (3%). Reducing the demand for these processes during torpor would reduce the demand for ATP, lower MR, and help to maintain metabolic balance. In addition, reduced energetic demand could account for reports of tolerance of hibernator tissues to hypoxia and ischemia (see *Potential Practical Lessons from Heterothermy*, later).

The maximal activity of Na^+/K^+ ATPase decreases in hibernation in several ground squirrel tissues (59, 183). These decreases may be due to reduced amounts of protein (59) or posttranslational modifications by phosphorylation (183). In jerboa kidney, however, the activity of this enzyme either increases, decreases, or remains unchanged in hibernation depending on which part of the nephron is examined (19). Na^+/K^+ ATPase activity can also be altered by changes in the membrane phospholipid environment in which it works (ref. 88, see next paragraph also).

Reducing Ca^{2+} ATPase activity might decrease ATP demand, and it appears that in skeletal muscle of arctic ground squirrels, the activity of sarcoplasmic reticulum Ca^{2+} ATPase decreases by half in the winter compared with summer, but there is no difference between torpid and aroused animals (185). Altering Ca^{2+} handling capacities has considerable implications for excitation-contraction coupling at low temperatures. Hibernator hearts maintain spontaneous contractions *in vitro* down to temperatures as low as 0°C (reviewed in ref. 154), even when sampled from euthermic animals. In contrast, nonhibernator hearts become arrhythmic below approximately 20°C and cease to beat around 16°C (53). For this reason, alone I believe that "human hibernation" at very low T_b will remain strictly in the realm of science fiction for the foreseeable future. Regulation of intracellular Ca^{2+} is key to maintaining excitability and contractility at low temperatures in hibernators (300). The mRNA and protein levels of Ca^{2+} ATPase from the left-ventricle is threefold higher in hibernating woodchucks compared with those of summer active conspecifics (310), perhaps facilitating improved Ca^{2+} handling. Beyond transcription and translation the activity of membrane-bound ion motive ATPases can be affected by the phospholipid environment in which they operate (e.g., ref. 88). During the fairly short time periods between phases of ground squirrel torpor bouts sarcoplasmic (224) and liver mitochondrial (10, 64), phospholipids are remodeled. The activity of Ca^{2+} ATPase in Syrian hamster heart increases as animals transition from IBE to entrance and torpor (Fig. 10A; ref. 121). This activity correlates positively and negatively with sarcoplasmic reticulum phospholipid content of the PUFAs linoleic acid (18:2, *n*-6) and docosahexanoic acid (22:6, *n*-3), respectively (Fig. 10B; ref. 116).

The activity of ion motive ATPases could also be downregulated *in vivo* by reducing the permeability of cell membranes across which ions move. Reducing ion leak occurs in other energy-stressed systems, including hypoxia-tolerant skeletal muscles of overwintering frogs and anoxia-tolerant turtle brains (reviewed in ref. 268). In the turtle brain, such decreases in ion permeability may be mediated by changes in the phosphorylation state of NMDA receptors (reviewed in ref. 51). The phosphorylation of NMDA1 receptors decreases in Arctic ground squirrel hippocampus between IBE and torpor (315) and hippocampal slices from torpid animals survive ouabain poisoning of Na^+/K^+ ATPase longer than those from conspecifics in IBE, suggesting a lower Na^+ and K^+ leak rate across plasma membranes (243). Despite this observation, hippocampal slices from torpid animals deplete ATP at a similar rate to those of interbout euthermic animals (63), arguing against a metabolic protective role of "channel arrest" in hibernation.

Protein synthesis accounts for up to 28% of cellular O_2 consumption (241), so ATP demand could be reduced considerably in temporal heterothermy if this cost could be reduced. Translation in liver is reversibly suppressed during daily torpor (78). In ground squirrel hibernation, protein synthesis is reversibly downregulated during arousal and the torpor phase in brain (105) and liver (289). The suppression of protein synthesis in hibernation appears to be due largely to low T_b blocking the initiation of "cap dependent" translation through phosphorylation-dependent inhibition of eukaryotic initiation factors (60, 292), along with inhibition of elongation (105). Recent evidence suggests that cap independent translation may proceed preferentially during arousal (222). Although protein ubiquitylation continues throughout ground squirrel torpor bouts (294), proteolysis is suppressed, likely due to low temperature (290, 293). Initiation of transcription is suppressed by half in hibernating golden-mantled ground squirrel, and mRNA elongation is very temperature sensitive, so that very little transcription is likely to occur at typical hibernation T_b (291).

Gluconeogenesis may be important to hibernators that rely on endogenous energy stores, because glucose is the preferred substrate for brain metabolism, so maintaining carbohydrate supply represents a significant metabolic challenge. In many ground squirrel, tissues enzymes for carbohydrate catabolism are downregulated in the transition from summer to winter (140). Posttranslational modifications that inactivate glycolytic enzymes in hibernation (for review, see ref. 273) and daily torpor (210) may delay the depletion of carbohydrate reserves. Nonetheless liver glycogen and glucose, as well as blood glucose, decrease within ground squirrel torpor

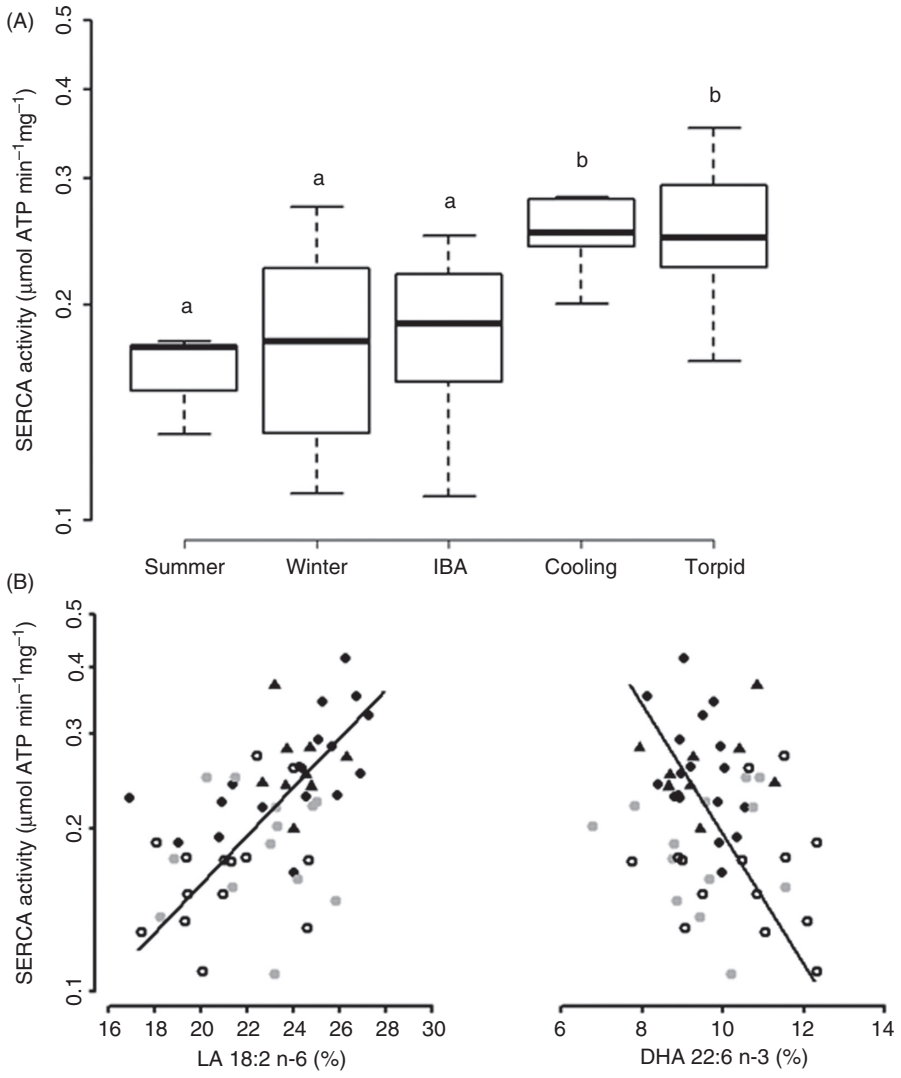

Figure 10 (A) Activity of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) in different phases of hibernation bout in Syrian hamsters. "Summer" and "Winter" animals were euthermic ($T_b \sim 37°C$). "IBA" = interbout arousal (equivalent to IBE), "cooling" = entrance. Groups differing significantly ($p < 0.01$, Tukey's post-hoc comparisons) are denoted by different superscripts. (B) The effect of cardiac sarcoplasmic reticulum phospholipid fatty acid composition on Ca^{2+} ATPase activity in different phases of a hibernation bout. Ca^{2+} ATPase activity as a function of the proportions (% of total fatty acids) linoleic acid (LA 18:2, *n*-6) (a), docosahexaenoic acid (DHA 22:6, *n*-3) (b), and (c) the ratio of LA/DHA. Black dots indicate data from torpid animals, black triangles from cooling animals, grey dots from animals during interbout arousals, and open dots from nonhibernating summer and winter Syrian hamsters. Modified from ref. 121.

bouts (6, 54, 109, 110, 253). Despite an early report in black bears (76), hibernators, like other mammals do not express key enzymes of the glyoxylate cycle (156) which can convert fatty acid derivatives into carbohydrate. Without dietary carbohydrates, temporal heterotherms might be expected to rely heavily on gluconeogenesis. Glycerol, derived from triglyceride lipolysis, may provide a ready precursor for glucose synthesis in hibernators. A more common scenario for starving animals is to hydrolyze muscle proteins and use the amino acids as carbon skeletons for gluconeogenesis. Such a strategy would risk atrophy, a condition to which hibernators may be resistant (see *Do Hibernators Resist Atrophy?*, later). Beyond the risk of atrophy, gluconeogenesis requires a significant investment in ATP, counter to the presumed benefits of heterothermy. It is not clear whether significant levels of gluconeogenesis occur during torpor, but liver glycogen levels are restored during arousal and IBE (113). This replenishment is probably facilitated by upregulated capacities for gluconeogenesis in hepatocytes isolated from golden-mantled ground squirrels exposed to cold, regardless of season or hibernation state (270). Moreover, in deer mice (*Peromyscus maniculatus*), a daily heterotherm, the activity of gluconeogenic enzymes are significantly reduced in torpor, but dramatically activated during arousal (208).

Although not related strictly to ATP demand, leak of protons through the inner mitochondrial membrane can account for a significant proportion of endotherm energy budgets, and could be a target for energy savings in temporal heterothermy. Pumping of protons across the IMM by the ETS develops the proton motive force, a chemiosmotic gradient that can be used to power ATP synthesis by the F_1F_O ATPase (i.e., ETS complex V; see Fig. 2). Under conditions of low ATP turnover, however, some protons may "leak" into the matrix across the IMM. This dissipation of the proton motive force stimulates the ETS to pump more protons and, in the process, consume more oxygen and energetic substrates derived either from exogenous or endogenous energy stores. Although the mechanism underlying this leak remains unclear, it accounts for up to 22% of endotherm energy budgets (242).

Despite the apparent energetic "waste" posed by proton leak, initial investigations in liver mitochondria showed no difference in IMM proton permeability between torpid and winter euthermic Arctic ground squirrels (16), or torpid and summer euthermic thirteen-lined ground squirrels (120), though dietary PUFA may affect this result (43). In a more recent study, we demonstrated that the transition between IBE and torpor actually results in an increase in proton leak, but only when measured *in vitro* at 37°C (38). When measured at 10°C, however, proton leak in torpor was significantly lower (38). During daily torpor in the dwarf Siberian hamster (*Phodopus sungorus*) proton leak does increase (40), perhaps as a mechanism to minimize an increase in reactive oxygen species production (43).

Beyond the classical view of a cellular energy budget, a recent study revealed a potentially unrecognized manner in which hibernators might reduce energy demand. Many proteomic changes occur in the heart of 13-lined ground squirrels over the course of the year. Though most of these changes are related to seasonal transitions at least one difference among phases of a torpor bout stands out with potential to conserve metabolic energy (122). Cofilin 2 (CFL2) is specific to muscle and regulates the turnover of actin filaments. Grabek et al. (122) found that phosphorylated CFL2 was virtually undetectable in torpor and early arousal, and at very low levels during entrance. During IBE, and indeed euthermia at any time of the year, phosphorylated CFL2 was quite abundant, and the total amount of CFL2 did not change throughout the year. When completely dephosphorylated CFL2 binds to actin, preventing ATP hydrolysis by actinomyosin ATPase, so dephosphorylating CFL2 during torpor may reduce ATP consumption in hibernator muscle.

It seems intuitive that heterotherms could save metabolic energy but reducing the cellular demand for ATP. Indeed some energy-demanding processes, including Na^+/K^+ ATPase and protein synthesis, appear to shut down during torpor. On the other hand, some metabolically expensive processes, including Ca^{2+} pumping and gluconeogenesis, are important for maintaining cellular and whole-animal physiological processes, especially at low T_b in fasting animals, and must remain active.

Turning down ATP production

While reducing the rate of ATP consumption is important to metabolic suppression, processes that generate mitochondrial membrane potential also have significant control over cellular oxidative metabolism (e.g., ref. 37), so the characterization of pathways that synthesize ATP is important for understanding how metabolic balance is maintained in heterothermy. ATP can be produced by oxidative phosphorylation or by substrate level phosphorylation through glycolysis. Important rate-controlling glycolytic enzymes, such as phosphofructokinase and pyruvate kinase, consistently show posttranslational modifications and decreased activities in hibernation (for review, see ref. 273). These changes are accompanied by changes in the enzymes likely responsible for phosphorylation—protein kinase C and A (273). Pyruvate dehydrogenase activity is downregulated by covalent modification in hibernating golden-mantled ground squirrel heart and kidney (36) and Djungarian hamster heart, BAT, and liver during daily torpor (134). Although such alterations probably serve to switch substrate preference from carbohydrate to lipid (see *Fuelling Temporal Heterothermy*, later) they may also restrict oxidative substrate supply to mitochondria.

Research into the effects of temporal heterothermy on mitochondrial oxidative phosphorylation began at least as early as 1966. These early studies often described conflicting results depending on the species under investigation, the tissue studied, the oxidative substrate supplied to the mitochondria and the *in vitro* temperature at which rates of oxygen consumption and/or ATP synthesis were measured (reviewed in ref. 267). However, many of these early studies compared animals in torpor with euthermic conspecifics in the summer, introducing potential seasonal complications including differences in photoperiod, reproductive states, and diet. How these differences might affect mitochondrial metabolism are of some interest, but are unlikely to be the best model for elucidating how acute metabolic changes are regulated, for example, during entrance into, and arousal from torpor. Recent studies attempt to control these variables by including comparison groups within the winter hibernation season. When summer groups are included, this approach allows the researcher to distinguish seasonal effects from acute effects that may change rapidly among different phases of a torpor bout.

Mitochondria isolated from the liver of torpid 13-lined ground squirrels exhibit state 3 respiration rates (near maximal respiration in the presence of saturating substrate and ADP) that are up to 70% lower than those from animals in IBE or summer euthermia (39, 200). A similar, if less extreme, pattern is seen in liver mitochondria of Djungarian hamsters (40, 167) and mice (42) that undergo daily torpor. In both daily torpor and hibernation, the suppression of liver mitochondrial state 3 respiration occurs with little change in state 4 respiration, a minimal respiration state with saturating substrate, but following the conversion of all of the added

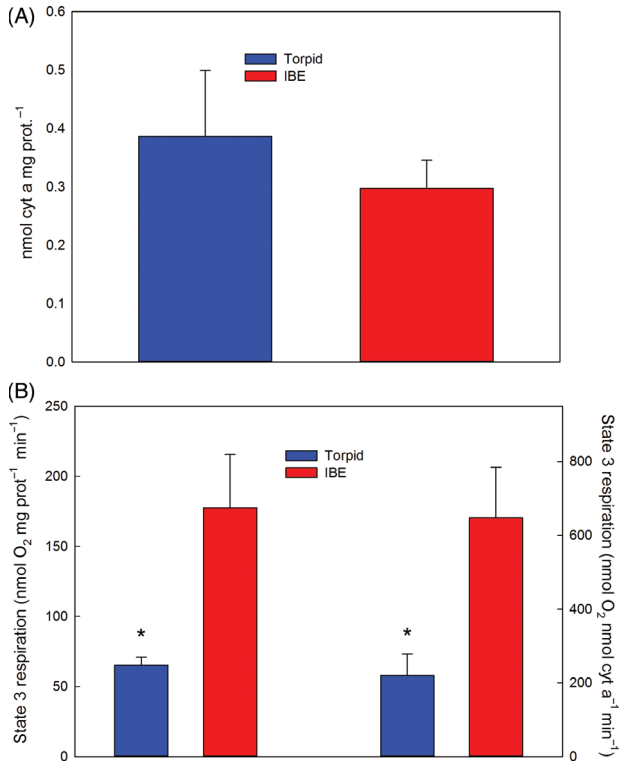

Figure 11 (A) The content of cytochrome *a* in liver mitochondria isolated from *Ictidomys tridecemlineatus* does not differ significantly between torpor and IBE. (B) State 3 respiration (with 10 mmol/L succinate as substrate plus 1 μmol/L rotenone and 0.2 mmol/L ADP) of liver mitochondria isolated from torpid ground squirrels is suppressed by approximately 65% whether expressed relative to mitochondrial protein or cytochrome *a* content. Asterisk indicates significant difference ($p < 0.05$, *t* test). K. Mathers, R. Balaban, J. Staples, unpublished data.

ADP to ATP. State 4 respiration is thought to support basal levels of mitochondrial proton leak.

Mitochondrial respiration rates are typically expressed relative to mitochondrial protein content, and it is possible that the suppression observed in state 3 is an artifact of changes in protein content between torpor and IBE. To assess this possibility one can also express respiration rates relative to mitochondrial quantities specific to oxidative phosphorylation. We found recently no difference between torpor and IBE in the content of liver mitochondrial cytochrome a (Fig. 11A) and, when state 3 respiration is expressed relative to this metric, the suppression in torpor relative to IBE remains (Fig. 11B).

The impressive degree of metabolic suppression seen in liver mitochondria has been reported in a diverse array of hibernators by several research groups (reviewed in ref. 267). However, such results depend on experimental conditions, and do not reflect the response in all tissues. In hibernators mitochondrial metabolic suppression in other tissues, such as skeletal muscle (16, 41) and cardiac muscle (39), is more modest (~30%). Brain cortex mitochondria exhibit no apparent suppression (108), though this result may depend on the method used to assess mitochondrial respiration (see *Estivating Ectotherms*, later). No apparent suppression of mitochondrial metabolism is seen in kidney, skeletal muscle, or heart during daily torpor in Djungarian hamsters (167). Several early studies examined mitochondria from hibernator BAT with varying results (reviewed in ref. 267) probably reflecting seasonal differences between summer and winter animals, rather than acute regulatory effects. To my knowledge, no comparison of BAT mitochondrial metabolism between torpor and IBE has been reported. Though such a study would be informative, I predict no difference between these two states, as I believe that BAT metabolism is regulated primarily through changes in T_{set} throughout a torpor bout (see *Endotherms in the Cold* and *Adjusting Thermoregulation*, earlier). Decreasing the lower limit of T_{set} below T_a (except for arctic ground squirrels that hibernate at very low T_a) during entrance into a torpor bout would halt sympathetic activation of BAT, so UCP1 would not be "open" and mitochondrial oxidative metabolism would be low. I predict, therefore, that any further downregulation of substrate oxidation or the ETS would not conserve significantly more energy. To some extent, this situation may be analogous to the relatively small metabolic suppression seen in skeletal muscle mitochondria; in a resting muscle, mitochondria are quite inactive (near state 4), so further suppression in torpor would likely have little selective advantage in terms of energy savings. On the other hand, the liver has a relatively small dynamic range for increases in MR (i.e. mitochondria operate closer to state 3, even at rest), so regulatory suppression of mitochondrial metabolism may have provided key energy savings over evolutionary time.

The pattern and degree of mitochondrial metabolic suppression also depends on other experimental conditions. Although dietary lipid quality alters whole-animal hibernation patterns (see *Food Quantity and Quality*, later), we found no effect of dietary PUFA on liver mitochondrial metabolism except with extremely high or low PUFA levels (120). The energetic substrate oxidized by isolated mitochondria also affects mitochondrial metabolic suppression; the greatest difference is seen with succinate but this difference is more modest with pyruvate (a product of carbohydrate metabolism) and glutamate (derived from amino acid metabolism; ref. 200). Heterotherms appear to alter oxidative substrate use during the transition between euthermia and torpor, reducing the relative contribution of carbohydrate and increasing that of lipid in torpor (see, *Fueling Heterothermy*, later). Despite this result we find no change in liver mitochondrial substrate preference between torpor and IBE, and low rates of oxidation with both pyruvate and palmitoyl carnitine (a fatty acid derivative) as substrates (200).

Experimental temperature also affects mitochondrial metabolic suppression in torpor, illustrating one complexity of designing hibernation experiments. The "native" temperature of mitochondria from ground squirrels in IBE is near 37°C but for torpid animals mitochondria function near 5°C. Ideally a range of physiologically relevant temperatures should be used to evaluate mitochondrial metabolism in heterotherms. Using such an approach we found that the greatest suppression

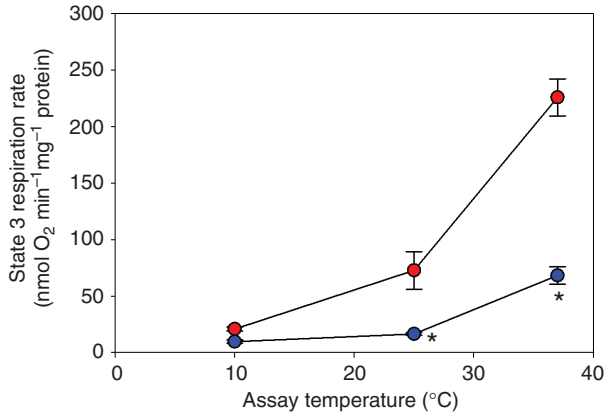

Figure 12 The effect of *in vitro* assay temperature on the respiration of liver mitochondria isolated from a hibernator, *Ictidomys tridecemlineatus*. At 37 and 25°C, state 3 respiration (in the presence of 10 mmol/L succinate, 1 µmol/L rotenone, and 0.2 mmol/L ADP) is significantly higher when isolated from animals in IBE (red) than in torpor (blue). Values are means of state 3 respiration. Asterisks indicate significant difference ($p < 0.05$, *t* test). Modified with permission from ref. 38.

between IBE and torpor in liver mitochondria occurs at 37°C, and is due largely to decreased substrate oxidation, probably through inhibition of succinate dehydrogenase (SDH, ETS complex II; ref. 38). At 25°C, suppression was still significant in torpor, though more modest, but at 10°C (the lowest temperature at which we could collect reliable *in vitro* data) respiration rates in torpor could not be distinguished statistically from IBE (Fig. 12; ref. 38). The decrease in temperature affected substrate oxidation, ADP transport and phosphorylation, and proton leak equally (38).

The differential effects of temperature on the *in vitro* performance of mitochondria inspired us to investigate the metabolism of mitochondria isolated from animals at different stages of a torpor bout. As shown in Figure 13, state 3 respiration increases ca. two-fold between torpor (T_b, 5°C) and early arousal (T_b, 15°C), and another twofold between early and late arousal (T_b, 30°C). Respiration does not peak until T_b reaches ca. 37°C in IBE (9). 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 late entrance (T_b, 15°C) or torpor (64). These findings suggest that rapid, active suppression of substrate oxidation may have a greater impact on whole-animal MR in the initial stages of entrance, before T_b falls substantially. Moreover, these data suggest that the mechanisms underlying this active suppression of mitochondrial substrate oxidation can only be reversed slowly during arousal, when T_b is initially low.

Transport of succinate into liver mitochondria does not appear to be differentially regulated between torpor and IBE in 13-lined ground squirrels (67). Moreover, the apparent affinity for succinate oxidation by intact mitochondria does not differ between torpor and IBE (39). These results suggest a down-regulation in maximal capacity for mitochondrial succinate oxidation, rather than changes in kinetics. Indeed we found significantly lower SDH activity in torpor, an effect that was due partially to inhibition of SDH by oxaloacetate, a Krebs cycle intermediate (9). This finding suggests that oxaloacetate

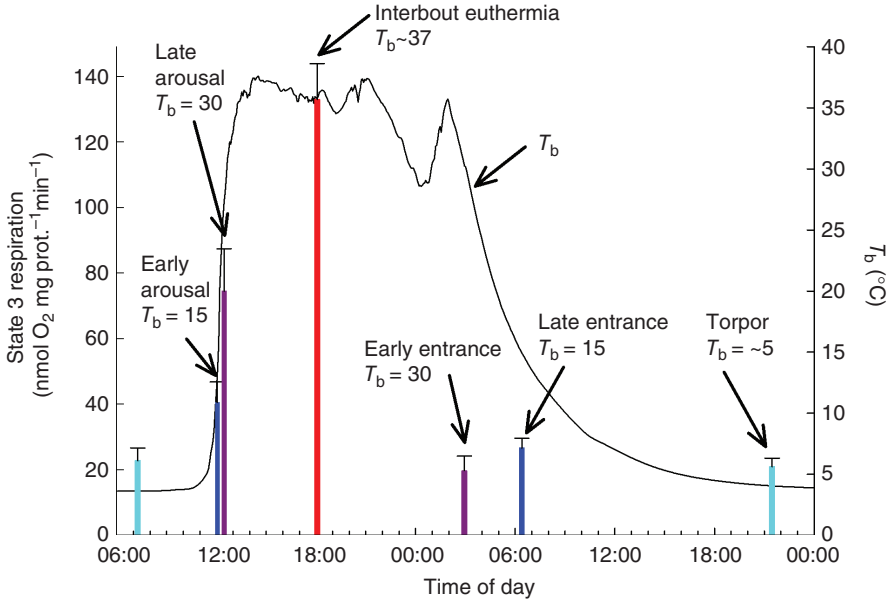

Figure 13 A representation of liver mitochondrial state 3 respiration rates (measured *in vitro* at 37°C with 6 mmol/L succinate, 1 µmol/L rotenone and 0.2 mmol/L ADP) during different stages of a typical torpor bout in *I. tridecemlineatus*. When mitochondria are isolated from animals in torpor, respiration is low. During arousal respiration increases, but not until IBE, where T_b is ~37 °C, does it reach maximal values. In contrast, respiration is rapidly and maximally suppressed in the early stages of torpor when T_b is still fairly high. Modified with permission from ref. 265.

accumulates within liver mitochondria during entrance and is cleared during arousal. A metabolomic study of whole liver in the same species did not report any such accumulation (206), though it is possible that changes in the low concentrations of mitochondrial oxaloacetate could not be detected by the methods employed. On the other hand, another study showed that liver concentrations of isocitrate, which counters the oxaloacetate inhibition of SDH, were lowest during torpor (94). Nonetheless, although oxaloacetate inhibition of SDH likely contributes to the suppression of liver mitochondrial metabolism in torpor, it cannot fully explain the 70% difference in state 3 respiration between IBE and torpor.

The dynamics of mitochondrial respiration throughout a torpor bout suggest that any mechanism underlying respiratory suppression is T_b sensitive (Fig. 13). When T_b is fairly high during the early stage of entrance, suppression occurs rapidly. Reversal of this suppression is relatively slow during arousal when T_b begins quite low. This temperature-sensitive pattern suggests that enzyme-catalyzed reactions underlie both the initiation and reversal of mitochondrial metabolic suppression. Enzymes responsible for posttranslational modification of mitochondrial enzymes, for example, by covalent phosphorylation or acetylation, could account for this observed pattern, assuming that such enzymes operate with typical Q_{10} values around 2.5. Indeed the phosphorylation of skeletal muscle phosphoglucomutase, a cytosolic glycolytic enzyme, changes among stages of a torpor bout (140).

Since the early 1990s, a role for posttranslational modification of intramitochondrial enzymes has received increased research attention. Soluble adenylate cyclase (sAC) has been identified within mitochondria (reviewed in ref. 288) and is stimulated to produce cyclic AMP (cAMP) in response to changes in several cellular conditions including changes in concentrations of ATP, Ca^{2+}, and HCO_3^-. This cAMP would stimulate intramitochondrial protein kinase A (PKA; ref. 249) to phosphorylate any of several ETS enzymes including cytochrome c oxidase (173), SDH (286) and F_1F_O ATPase (227). It is possible that changes in hepatocyte ATP, Ca^{2+}, or HCO_3^- during entrance into a torpor bout could trigger a phosphorylation cascade that would downregulate liver mitochondrial metabolism. This possibility is especially intriguing for HCO_3^- because entrance into a torpor bout is associated with a retention of whole-body CO_2 (reviewed in ref. 197) presumably accompanied by an acidosis (at least relative to the pH of neutrality, which increases as temperature decreases). Despite this intriguing possibility no changes in the phosphorylation of liver mitochondrial proteins have been reported, though seasonal differences are evident (65). A more recent proteomic study of whole liver showed few changes in posttranslational modifications among stages of a torpor bout (139). It is possible, however, that the techniques employed in this study were not sensitive enough to detect posttranslational modifications in mitochondrial oxidative phosphorylation proteins, which account for only a small fraction of the total liver proteome.

Three protein deacetylases, namely sirtuins, that is, SIRT3, SIRT4, and SIRT5, are found within the matrix of mammalian mitochondria (reviewed in ref. 3). In nonhibernators fasting increases liver mitochondrial SIRT3 expression, leading to deacetylation of long-chain acyl coenzyme A dehydrogenase, presumably facilitating mitochondrial fatty acid oxidation (142). Ground squirrels do not eat throughout the winter so it is surprising to find that the hibernation season is associated with a decrease in liver SIRT3 levels and a general increase it protein acetylation (139). SIRT3-mediated deacetylation alters the activity of SDH (66), a result that is particularly relevant to hibernators given the 70% suppression of succinate oxidation in torpor. However, no such specific acetylation changes were noted in whole ground squirrel liver among different seasons or stages of torpor bouts, although subunits of the F_1F_O ATPase may be differentially acetylated (139). Again, it would be informative to repeat such proteomic studies using mitochondria purified from the livers of animals in different torpors states. Such mitochondrial studies would not only improve resolution of candidate proteins but permit correlation of acetylation state with mitochondrial function for preparations from the same individual animals.

In summary, it appears that ATP production capacities are reversibly suppressed during heterothermic periods. Mitochondria isolated from torpid heterotherms show dramatic reductions in metabolic rates during maximal ATP synthesis. The degree of mitochondrial metabolic suppression also depends on the temperature at which assays are performed. This suppression appears to be initiated quickly and early during the entrance phase, but is reversed only slowly during arousal, suggesting a T_b-sensitive, enzyme-mediated process. The significance of this mitochondrial metabolic suppression to whole-animal MR remains in question, however, as it is not seen to the same degree in all tissues, and is generally greatest when succinate is used as substrate. Succinate is likely an uncommon substrate *in vivo*, especially compared with catalysis products of carbohydrates (e.g., pyruvate), fatty acids (e.g., palmitoyl carnitine) or amino acids (e.g., glutamate), which, when supplied to mitochondria as substrates, generally demonstrate more modest metabolic suppression in torpor.

Quantitative contributions to energy savings

While it is clear that the reduction of MR in temporal heterothermy can be caused by reducing T_{set} and thermoregulatory heat production, passive thermal effects as T_b falls and active suppression of nonthermogenic metabolism, little data exist to allow us to estimate the relative contributions of these three effects. What is obvious is that even at a fairly high T_a, small mammals must invest considerable energy in thermogenesis. For example, euthermic golden spiny mice (*Acomys russatus*) have a MR rate that is a least twofold higher when housed at 23°C compared with conspecifics housed at thermoneutral temperatures (approx. 30°C; ref. 123). Simply "turning off" thermogenesis would reduce MR by at least

50% without passive thermal effects or active suppression of nonthermogenic metabolism. Unfortunately early studies analyzing the relationship between T_b and MR, especially during entrance, often conflated this thermoregulatory effect with active metabolic suppression; Q_{10} values greater than 3 were used to argue in favor of active, regulated metabolic suppression, without considering the reduced T_{set} during entrance and thermal inertia of the animal.

It is evidently difficult to quantify the contribution of thermogenesis to total MR in an animal living at low T_a, but some unique small mammals display heterothermy even at thermoneutral temperatures. For example, the edible dormouse, *Glis glis*, will enter daily torpor under thermoneutral conditions (T_a, 28°C). Under these conditions, entrance into torpor is accompanied by a rapid decrease in MR, before any decline in T_b. Perhaps more impressive is the daily torpor displayed by the golden spiny mouse. When deprived of food these small mammals reversibly enter torpor, even when held at a T_a, of 35°C. Under these conditions, MR is reduced by approximately 25%, presumably by active mechanisms in nonthermogenic tissues that are temperature independent, because T_b changes minimally (123).

As mentioned earlier, other evidence supporting active metabolic suppression comes from studies that compare mitochondria isolated from torpid and IBE hibernators. In ground squirrel liver the maximal degree of suppression is approximately 70% (39). In mice, the liver accounts for 6.2% of body mass but 18.5% of the total metabolism of all tissues (187). If a similar relationship holds for ground squirrels, suppression of liver mitochondrial metabolism alone could reduce whole-animal MR by over 5% with no change in T_b. The maximal mitochondrial metabolic suppression in skeletal muscle is more modest, approximately 30% (41). However, this tissue accounts for up to 30% of total tissue metabolism in mice (187), so this level of suppression could reduce whole-animal MR by 9%. Suppressing mitochondrial metabolism in these two tissues alone may account for more than half of the whole-animal, T_b-independent MR suppression seen in heterotherms under thermoneutral conditions. If T_b subsequently fell from 37 to 5°C passive thermal effects would further decrease MR to 5% of its original levels (assuming a Q_{10} of 2.5), close to what is observed in hibernating ground squirrels (Fig. 8).

Fuelling temporal heterothermy

Temporal heterothermy is presumed to provide a selective advantage by conserving energetic reserves over the time of year when this food energy availability is at its lowest. Suppression of metabolism on its own, however, would likely be insufficient to fuel the entire winter without alterations in the patterns of foraging and/or eating, so in preparation for the heterothermic period these animals alter both the quantity and quality of the food they ingest or store. Although many hibernators fast throughout the winter, they do not typically exhibit classic symptoms of starvation such as high blood urea nitrogen indicative of accelerated protein catabolism (reviewed in ref. 56). Moreover skeletal muscle appears to be remarkably resistant to catabolism in hibernation (see *Do Hibernators Resist Atrophy?*, later). These observations lead to the conclusion that the reduction in metabolic rate allows for endogenous energy stores, especially lipid, to fuel the hibernation season in those species that do not cache food. This conclusion is based largely on laboratory observations, however, and in the wild overwinter mortality in Columbian ground squirrels (*Urocitellus columbianus*) may exceed 60% (202). Higher body masses (and, presumably, greater lipid stores; see Fig. 5) correspond with improved overwinter survival (202), so the acquisition of sufficient energy stores was likely a significant selective pressure in the evolution of temporal heterothermy, especially hibernation.

Food quantity and quality

Under captive conditions, adult obligate hibernators dramatically increase food intake from mid-spring to early autumn. A corresponding decrease in resting MR as summer progresses results in body mass peaking just prior to the beginning of hibernation (Fig. 14). In the wild, however, changes in resting MR have little effect on fattening in Arctic ground squirrels, and males and females reach peak body mass with 52 and 62% fat, respectively, but only slight increases in lean body mass (255). These results are, perhaps, not surprising in a hibernator that relies on endogenous energy stores considering that lipid provides the most energy-dense metabolic substrate, compared with carbohydrate of protein. The increase in food intake and adiposity are accompanied by dramatic increases in the activity of enzymes involved in fatty acid and triglyceride synthesis in golden-mantled ground squirrel white adipose tissue (299).

The sustained increases in body fat suggest that regulation of summer food intake in hibernators differs from nonhibernating mammals. For the most part evidence suggests that hibernators respond to leptin in similar ways to nonhibernators (reviewed in ref. 97), though in prehibernatory little brown bats (*Myotis lucifugus*) leptin secretion actually decreases as body fat increases (165). Along with leptin secretion and circulating levels, the expression and quantity of leptin receptors in the hypothalamus have considerable control over food intake but, to my knowledge, no data are available about this subject over the annual cycle of a hibernator. Other signaling pathways known to be important in regulating mammalian food intake, such as insulin, ghrelin and AMPK also seem to be regulated differently in hibernators, but their roles have not been conclusively established (reviewed in ref. 97). Recent data show that hibernating grizzly bears respond to insulin in the spring and autumn, but not during the hibernation season (207). Clearly, this is a fruitful area for future research. Unfortunately, I am aware of no comparable data regarding daily heterotherms, except to note a potential effect of AMP in initiating fasting-induced daily torpor (see *Regulation of Temporal Heterothermy*, later).

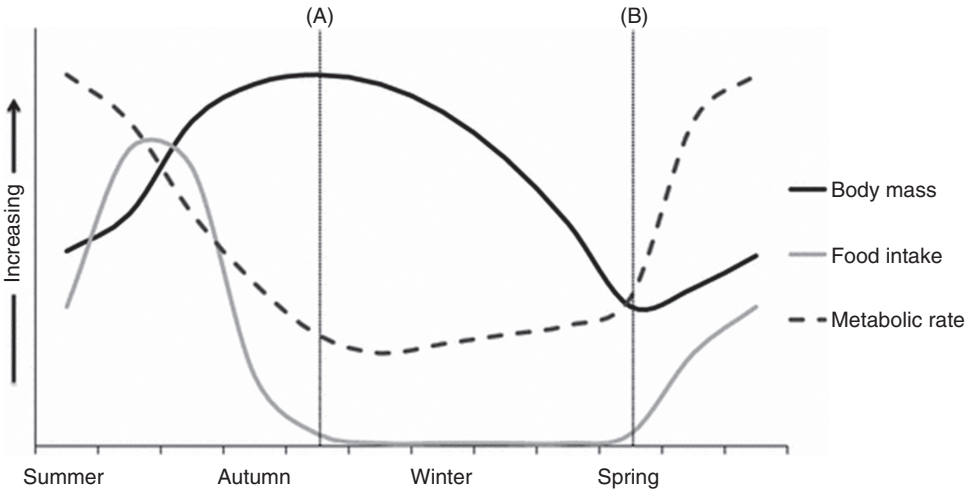

Figure 14 Schematic of circannual cycle of body mass, food intake, and metabolism in a lab-maintained rodent hibernator. Taken from ref. 97, with permission.

Beyond the quantity of food consumed over the warm months, obligate hibernators also appear to regulate the quality of the food they consume. As summer progresses, ground squirrels switch from being primarily herbivores to increasing the proportion of grains in their diet. This switch corresponds with an increase in the dietary content of essential PUFA, which are incorporated into depot fats and membrane phospholipids (102, 103, 131). Laboratory studies show that diets supplemented with moderately high linoleic acid (18:2, *n*-6) result in a higher proportion of animals entering torpor, lower torpor T_b and longer bout duration during hibernation or daily torpor in chipmunks (119), deer mice (114), and golden-mantled ground squirrels (101), but not 13-lined ground squirrels (120). In the wild, a diet containing moderate PUFA levels (33-74 mg/g) improves the ability of Arctic ground squirrels to survive the hibernation season, compared with diets containing more than 74 mg/g of PUFA (103). The moderate PUFA diet also corresponds with longer torpor bouts, fewer arousals, more days spent torpid, and shorter combined durations of arousal, IBE and entrance (103). Incorporating PUFA into phospholipids increases fluidity, allowing membranes to remain in the liquid crystalline state required for proper function, down to lower temperatures. The carbon-carbon double bonds present in unsaturates are, however, targets for peroxidation when exposed to reactive oxygen species, presumably imposing an upper limit on any selective advantage to increasing PUFA incorporation into hibernator lipids. To my knowledge, however, mechanisms by which hibernators change preference for dietary lipid throughout their feeding period remain completely unknown.

In the Djungarian hamster, a daily heterotherm, acclimation to long photoperiods, and cool T_a increases preference for dietary unsaturates (138), and a diet rich in PUFA increases torpor expression (118). In the same species, it has recently been demonstrated that dietary cholesterol is also required for expression of daily torpor (152).

Fuel use

In general, the heterothermic period appears to be associated with a shift away from carbohydrate oxidation and toward lipid oxidation. Beyond respirometry data (see next paragraph), evidence supporting this pattern comes from studies on gene expression (52), protein levels (93, 140), enzyme compartmentation (129, 132, 210, 277), and posttranslational modification (36, 134, 272). This suppression of carbohydrate catalysis is very effective—even though ^{13}C-labelled glucose is transported into heart and brain cells of torpid and aroused 13-lined ground squirrels, virtually none of it is oxidized (6).

Respirometry studies indicate that in both hibernation (Arctic ground squirrel, ref. 47; golden-mantled ground squirrel, ref. 258; black bear, ref. 2) and daily torpor (deer mice, ref. 209) whole-animal MR in the torpor phase is fuelled almost entirely by lipid metabolism. In arctic ground squirrels, as T_a, falls from 2 to −12°C the respiratory exchange ratio (RER; CO_2 excretion/O_2 consumption) in torpor increases from 0.72 to 0.82 (157), suggesting increased carbohydrate or protein oxidation, even though shivering is not seen below 5°C (157). In arousal, the initial phases, at least, appear to be fueled largely by lipid oxidation in Arctic ground squirrels (157). In golden hamsters most of the circulating lipid appears to be derived from BAT (204), and some of this BAT-derived lipid may fuel other tissues (193). In golden-mantled ground squirrels, however, early arousal is associated with RER values >1 (258), suggesting that the animals are not in steady state. It has been hypothesized that this high RER represents the reversal of CO_2 retention associated with entrance into torpor in hibernators (20) and daily heterotherms (209); however, this pattern may also be explained by changes in CO_2 solubility of tissues as T_b changes (89). For a more thorough treatment of ventilation and gas exchange in hibernation, consult this overview article (197). In some hibernators, lactate levels increase up to 15-fold in heart and skeletal

muscle (big brown bats; ref. 70) and 8-fold in plasma (thirteen lined ground squirrels; ref. 212) during arousal, suggesting that intense shivering recruits anaerobic glycolysis. This conclusion has been challenged, however, by recent data that show blood lactate during arousal increases only to levels typical of euthermia (94). Regardless, any excess lactate is probably transported to the liver where it would serve as a gluconeogenic substrate.

In preparation for winter, one might predict that heterotherms would increase their capacity for lipid mobilization, transport and oxidation. Indeed expression of hormone-sensitive lipase (HSL) in marmot white adipose tissue increases in the winter (306). Pancreatic triglyceride lipase protein levels are also upregulated in heart and white adipose tissue of thirteen lined ground squirrels (260). Moreover norepinephrine stimulated lipolysis is higher in white adipose tissue from torpid thirteen lined ground squirrels than summer, euthermic animals, especially when measured at 4°C, close the torpid T_b (233). Taken together, these data suggest increased capacities for lipid mobilization from storage tissue during the hibernation season. The response does not appear to be universal among hibernators, however, as white adipose tissue HSL activity does not differ between torpor and euthermia in prairie dogs (100).

Once mobilized from white adipose tissue triglycerides, fatty acids are transported through the blood bound to albumin. In the winter hibernation season, the plasma albumin concentration increases by ~50% in wild black bears (172) and Richardson's ground squirrels (261). Once transported to peripheral tissues, fatty acids bind intracellularly to fatty acid binding proteins (FABP). Hibernation is associated with increased mRNA for FABP-coding genes as well as FABP protein content in BAT, heart, and skeletal muscle of 13-lined ground squirrels (143) and big brown bats (86), respectively. A more thorough examination of the annual cycle of one hibernator (13-lined ground squirrel) reveals that, in WAT, changes in FABP are strongly influenced by season but not hibernation status (i.e., peaking in winter), though torpor bout length does influence abundance of this protein (141).

Within the cell, transport of fatty acids into the mitochondrial matrix is facilitated by carnitine acyl transferases. In the little brown bat protein levels of one such enzyme, carnitine palmitoyl transferase 1, doubles in BAT between arousal and torpor, but does not change in skeletal muscle (85). This effect may be species specific, however, as mRNA levels of two isoforms of this enzyme increase from spring to autumn, but do not differ between torpor and IBE in BAT from the thirteen lined ground squirrel (126). Similar expression patterns are found for ground squirrel BAT nuclear genes coding for enzymes of β-oxidation within mitochondria (126). Similarly, enzymes participating in fatty acid oxidation are substantially upregulated in ground squirrel skeletal muscle (140) and liver (139) between summer and winter, with little difference among stages of torpor bouts. Despite these observed changes, oxidation of palmitoyl carnitine, a 16 carbon saturated fatty acid-derived substrate, by mitochondria isolated from 13-lined ground squirrel liver and skeletal muscle does not change between summer and winter, or between torpor and IBE (200). These results may indicate that changes in lipid mobilization and transport are more important for supporting lipid metabolism during the hibernation season than are changes in oxidation pathways.

Although dietary PUFA can certainly influence the composition of cellular membrane phospholipids (see previous section), mitochondrial membrane phospholipids are dynamically remodeled among different phases of a torpor bout (11, 64) and throughout the hibernation season (13). Such diet-independent changes might reflect differential mobilization of lipids, especially PUFA, at different stages of the circannual cycle. It certainly appears that essential PUFA are preferentially reserved in liver, depot (white) adipose tissue, and BAT over the hibernation season (reviewed in ref. 99). This pattern conflicts with data from nonhibernator studies of whole-animal and isolated adipocytes which demonstrate that fatty acids with more double bonds are preferentially mobilized, probably because their relatively greater water solubility allows more contact with lipases. In contrast, we recently found that unsaturates, especially 18:1, *n*-9 and 18:2, *n*-6, are preferentially retained in adipocytes from either summer active or winter torpid thirteen lined ground squirrels, whether measured at 4 or 37°C (233).

When hepatocyte β-oxidation produces more acetyl CoA than can be used by the Krebs cycle, some is diverted to ketogenesis. Ketone bodies can be oxidized by neurons so, with the constraints on carbohydrate supply in non-food-storing hibernators, ketones may be an important brain fuel in hibernation. Seasonally, the ketone body β-hydroxybutyrate increases in the blood of thirteen lined ground squirrels during the winter, peaking during torpor (6). Increased ketogenesis is likely facilitated by upregulated liver protein levels of ketogenic enzymes (139). Within a torpor bout, however, the blood concentrations of another ketone body, acetoacetate, change dynamically, with low levels in torpor and arousal, and threefold to fourfold increases in IBE and entrance (92). These oscillations suggest that ketogenesis is inhibited at low temperatures, but ketogenic enzyme activity may also be differentially regulated among torpor bout phases by posttranslational acetylation (139). Ketone body uptake is facilitated by an increase in the ground squirrel blood-brain barrier MCT1 transporter during the hibernation season (6). Although these data suggest increased reliance on ketone body for oxidative metabolism, mitochondrial oxidation of β-hydroxybutyrate in ground squirrel heart and brain does not change between torpor and IBE (108). Moreover liver mitochondrial oxidation of β-hydroxybutyrate is actually highest in summer than winter, with no difference between torpor and IBE (200). Again, these results suggest that differences in production and/or transport of ketone bodies exert the most control over their oxidation *in vivo*.

Heterotherms prepare for low energy seasons by altering the quantity and quality of the food they eat, but the mechanisms by which feeding behavior and adiposity are regulated

are not well understood. The increased food intake is paralleled by seasonal increases in capacities for triglyceride synthesis. Carbohydrate use appears to be acutely decreased during torpor. On the other hand, pathways for lipid mobilization and transport appear to be regulated seasonally, though capacities for mitochondrial lipid oxidation do not appear to change throughout the year. Taken together, these adjustments suggest a reliance on energy-dense lipid oxidation to fuel the heterothermic season. At the same time, increased ketone oxidation and reduced carbohydrate use throughout this period likely minimizes the requirement to degrade muscle protein to provide substrates for gluconeogenesis.

Regulation of temporal heterothermy

Elucidating how hibernation and daily torpor are regulated is of profound interest to both fundamental and applied biologists. Understanding how an animal can reversibly reduce MR and T_b will provide insights into mechanisms that govern oxidative metabolism, an area that has been debated at least as early as 1955 when Britton Chance and his colleagues published their groundbreaking findings (58). Moreover, manipulating that regulation, allowing metabolism to be reduced "on demand" has tremendous implications for biomedical applications such as improving survival of traumatic injury. Many researchers have been attracted to the appealing idea that a single compound can orchestrate the complex physiological and metabolic changes associated with heterothermy. Despite considerable effort the identity, or even existence, of a "hibernation induction trigger" (HIT) remains in doubt. This search is also complicated by the fact that hibernation in most animal models only occurs in the winter and is accompanied by fasting, so changes related specifically to metabolic suppression may be masked by effects of photoperiod, ambient temperature, reproductive status, and food intake.

How is torpor induced?

As reviewed earlier in this series (296), blood plasma or serum taken from hibernating thirteen lined ground squirrels induced hibernation when infused intravenously into summer animals of the same species that normally do not hibernate. Sometimes, however this induction required several days after infusion while the animals were housed in winter-like, cold and dark conditions (77, 244). A subsequent study (295) that employed careful controls found that plasma from hibernating Richardson's ground squirrels induced summer hibernation when infused into thirteen lined ground squirrels, but not when injected into Richardson's ground squirrels. Moreover infusions of saline were at least as effective as hibernating plasma at inducing summer hibernation in thirteen lined ground squirrels (295). Such results do not support the existence a blood-borne HIT, but rather suggest that thirteen lined grounds squirrels are a poor bioassay model for evaluating potential HIT. Despite these results the search for such an HIT continued, and it has been purported to be present in several hibernators, including woodchucks, bats, black bears (216) and even polar bears (44).

Subsequent studies, which attempted to purify HIT from the plasma of hibernators, suggested that it was a heat-labile peptide with a molecular weight less than 5 kDa (215) and was closely associated with the plasma albumin fraction (217). When this albumin fraction from woodchucks was injected into the cerebral ventricles of macaques (*Maccaca mulatta*) it induced symptoms such as hypothermia, bradycardia and aphagia (218). These effects were blocked by opiate antagonists, suggesting that hibernator plasma contains an endogenous opiate (218). Further studies suggested that D-Ala2-D-Leu-Enkephalin (DADLE), an endogenous δ-opioid, could induce summer hibernation when injected intracerbroventricularly, but μ- and δ-opioids were ineffective (216). It seems clear that changes in the brain opioid systems do occur in hibernators (214, 221, 296), but I believe that the significance for regulating hibernation remains obscure. Nonetheless, the potential role of this system remains intriguing, especially since hibernator plasma appears to improve the ability of skeletal muscle to survive hypoxia (146), though protection of heart may require several days of pretreatment (26).

Entry into torpor is accompanied by changes in the levels of many hormones and neurotransmitters known to be important in metabolic regulation (reviewed in ref. 303); however, these correlations have not led to any definitive demonstrations of causation. Surprisingly, inhibition of the parasympathetic nervous system does not affect daily torpor expression in Djungarian hamsters, but inhibition of the sympathetic nervous system does (31). These results confirm earlier data using knockout mice models known to enter fasting-induced torpor; dopamine β-hydroxylase knockout mice, that are incapable of synthesizing norepinephrine, do not express torpor (279). This effect can be overcome in ob/ob knockouts that are also deficient in leptin (279), offering an intriguing insight into potential mechanisms. The use of knockout techniques in mice, a species that expresses fasting-induced torpor, is a powerful experimental approach that offers promise for determining mechanisms that underlie torpor induction.

Several endogenous compounds important to metabolic regulation display concentration cycles that parallel patterns of hibernation and torpor making them candidates for roles in inducing torpor (reviewed in ref. 194). Other studies of metabolite profiles, however, suggest that many of the changes seen in hibernators are associated with seasonal changes, rather than the reversible transition between torpor and IBE (92). Many of these changes are associated with fasting and increased reliance on lipid metabolism associated with hibernation. Nonetheless there appear to be some specific plasma metabolite "biomarkers," some of which are listed in Figure 15, that differ among stages of a torpor bout (92). Whether these metabolites are involved in torpor induction or simply reflect changes in metabolic pathways following induction is not yet known.

It has been hypothesized that activation of adenosine 5′-monophosphate (AMP)-activated protein kinase is important

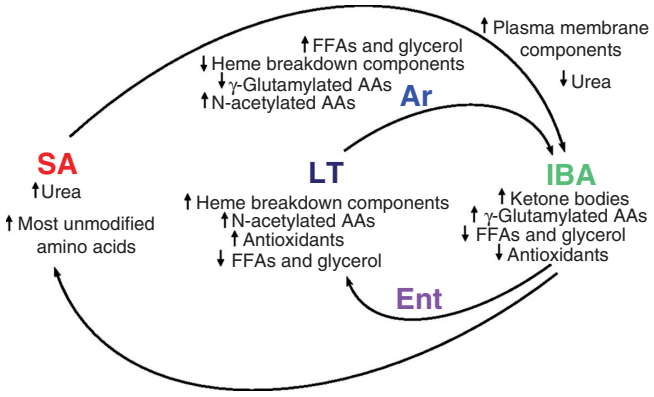

Figure 15 Schematic representation of changes in plasma metabolite concentrations between seasons and hibernation states in thirteen lined ground squirrels. Clusters of plasma metabolites shift in abundance with the nested metabolic cycles of hibernation. Sampling point abbreviations; SA, summer active; IBA, interbout aroused (equivalent to IBE); Ent, entrance into a torpor bout; LT, late in a torpor bout; Ar, arousal. Other abbreviations; AAs, amino acids; FFAs, free fatty acids. From ref. 92, used with permission.

for inducing torpor, especially given the fasting associated with torpor and hibernation, because ATP/AMP is thought to be a powerful indicator of cellular energy status (194). While injection of AMP into mice does induce hypothermia the rate of T_b decline is faster than that exhibited in fasting-induced (278) or workload-induced torpor (276). Moreover, the decline in MR induced by AMP could be explained entirely by passive thermal effects as T_b fell, rather than active metabolic suppression (276). Other signals of cellular energy status may hold more promise for inducing torpor. For example, ground squirrel liver concentrations of NAD^+ increase during entrance into a torpor bout (253), potentially serving as a signal that could activate NAD-dependent deacetylases, the SIRT. Such activation could, in turn, activate fibroblast growth factor 21 (FGF21), a hormone that promotes lipolysis. Indeed circulating levels of FGF21 increase substantially during the hibernation season, especially during IBE, but overexpression of FGF21 did not induce torpor in thirteen lined ground squirrels that were outside of the normal hibernation season (205). Nonetheless this study (205) is the first, to my knowledge, to use a (transiently) transgenic hibernator, and points the way toward a powerful research tool in the field.

Hydrogen sulfide is a potent inhibitor of cytochrome c oxidase, a powerful signaling molecule, and can be produced endogenously from cysteine by the action of such enzymes as cystathionine γ-lyase, cystathionine β-synthase, and 3-mercaptopyruvate sulfurtransferase. These properties make H_2S an intriguing candidate for inducing torpor and metabolic suppression. Indeed exogenous administration of H_2S gas induces a torpor-like state in mice, reducing MR by approximately 90% and T_b close to ambient temperature down to 5°C over 6 h (24). Unfortunately, this study (24) only used T_a below the TNZ of mice (~28°C), so the effects of H_2S may be due simply to disruption of thermogenesis. The role of H_2S in torpor has also recently come into question, as exposure of sheep to similar concentrations of H_2S used on mice does not alter significantly MR or T_b (130). Because H_2S is very reactive, it has been difficult to follow tissue levels in heterotherms, but recent advances in analytical techniques have allowed researchers to compare blood H_2S and its metabolites between hibernation and summer activity in grizzly bears (239). The results suggest that "recycling" of H_2S from its oxidative products may increase during hibernation, perhaps indicating a role in signaling or metabolic suppression while preserving cysteine for synthesis of reduced glutathione, an important antioxidant.

At least as early as 1992 (e.g., ref. 261) researchers have been interested in a potential role for differential gene expression in the regulation of hibernation and torpor. This field has been reviewed fairly recently (5) and since that time, further analyses of gene expression (309) and protein profiles (139) suggest that, like plasma metabolites, most of the changes are associated with fasting and environmental seasonality. In 1992, however, the presence of a hibernation-specific protein complex started to be reported in Asian chipmunks (*Tamias sibricus*). Three of the four "HP" proteins belong to the C1q and tumor necrosis factor superfamily, are produced exclusively in the liver, and are secreted into the blood plasma where they form heterogenous oligomers (163, 280). Subsequent studies demonstrated that plasma HP levels cycle seasonally, rising from a nadir in summer to a peak near the middle of the hibernation season (164). Moreover, within the hibernation season the increase in concentration of HP in cerebrospinal fluid precedes the decrease in T_b as animals enter torpor, and blocking HP action using antibodies decreases the amount of time chipmunks are torpid (164). This same research group reported recently that, within the rodent Scuirid family, HP is expressed exclusively in species that hibernate (251). Taken together these results were interpreted to suggest that HP serves as a circannual and, perhaps, acute hormonal signal triggering hibernation. More recently, however, the HP gene has been shown to exist broadly within placental mammals including nonhibernators such as dolphin but, curiously, not in mouse which undergoes fasting-induced daily torpor (252). In domestic cattle, that do not hibernate, the HP shares many molecular properties with that from the hibernating chipmunk and even oscillates seasonally in a similar way (252). From a functional standpoint, injection of recombinant cow HP into mice cerebral ventricles reduces food intake, but does not alter MR or T_b (252). Such results might be expected to deflate efforts to seek a role for differential gene expression to regulate torpor. Recently, however, it was found that both fasting-induced and spontaneous daily torpor are associated with strong induction of thioredoxin-interacting protein (Txnip) in both central and peripheral tissues (127). This observation is intriguing given the role of Txnip in energy sensing and, perhaps, its contribution to the regulation of oxidative metabolism.

What "Causes" arousals?

In some respects, the topic addressed in this section and the preceding section may be considered two sides of the same coin—if torpor is induced by the accumulation of a metabolite or gene product, arousal will be triggered when the concentration of this compound falls below a threshold. While such a scenario is certainly possible, further examination reveals that the "need" to arouse may be related to several other factors. The ability to employ temporal heterothermy is assumed to be an adaptation to conserve energy during periods of environmental stress. As such one might reasonably predict that, during the heterothermic season (usually the winter), animals would maximize the time spent torpid, and minimize MR and T_b. A corollary to this prediction is that heterotherms should minimize the frequency and duration of arousals and IBE, which account for the vast majority of energy used during the heterothermic season (298). This pattern appears to hold for small heterotherms, where allometry limits the amount of endogenous energy they can store. Within hibernators, however, these predictions are not always supported.

Intraspecifically among hibernators, larger (and, presumably, fatter) animals spend less time torpid, spend that time at higher T_b (313), and arouse more frequently (21). Contrary to the prediction that heterotherms should maximize energy conservation, these patterns suggest that, if energy stores allow, heterotherms minimize the amount time spent torpid. This pattern corresponds with observations from food-caching heterotherms such as Eastern chipmunks, but appears to conflict with most observations of enhancing dietary PUFA in fat-storing hibernators (see *Obligate vs. Facultative Hibernators*, earlier). Nonetheless, this pattern has been described as the "hibernation optimization hypothesis" (30), and implies that time spent torpid has potentially negative consequences that should be minimized by frequent arousals, when energy reserves permit. While some of these negative consequences may be ecological, such as the inability of torpid animals to avoid predation, the duration of IBE periods correlates strongly with the MR during the IBE (104), implying that arousals are necessary to increase MR to rectify some metabolic or physiological imbalance incurred during torpor.

The nature of the physiological imbalances that require arousal have been reviewed previously in this series (296). At that time, potential contributing factors included the requirement to enter slow wave sleep, which is not achieved while torpid (71). Since then numerous studies have shown that neuronal synapses in various parts of the brain regress during torpor, potentially serving a neuroprotective role against low T_b. This process is likely mediated by hyperphosphorylation of tau, a microtubule associated protein, but prolonged regression may lead to neuropathies, so arousal may serve to reverse temporarily the tau hyperphosphorylation and synaptic regression, minimizing neuronal damage in hibernators (reviewed in ref. 7).

Arousal from torpor may also be necessary to activate the immune system. Administration of bacterial lipopolysaccharide to torpid golden-mantled ground squirrel elicits no acute-phase response or fever, but does induce a fever during the subsequent IBE, prolonging its duration by several hours (232). These results suggest that arousal is necessary to cope with infections that might accumulate during torpor. This requirement may be especially important for infection by *Pseudomonas* species which grow well even at temperatures typical of torpor in small mammals (181). Supporting the importance of immune function, data from a hibernator (Syrian hamster) and a daily heterotherm (Djungarian hamster) show that low T_b during torpor reduces circulating lymphocyte levels, an effect that is completely reversed upon arousal (28).

The search for the "holy grail" of a HIT appears to continue, although evidence supporting its existence is scant. Many blood metabolites appear to change among phases of a torpor bout, but these changes may simply reflect underlying changes in metabolic pathways as opposed to signals *per se*. It is clear that changes in several important signaling systems, including δ-opioids, FGF21, H_2S, and Txnip, are associated with hibernation, but definitive demonstrations of causation are lacking. In this regard, model heterothermic species for transgenic manipulation, such as mice and thirteen lined ground squirrels, may prove to be very strong experimental tools. Equally enigmatic is the "cause" of periodic arousals in hibernators, though the role of potentially harmful tau hyperphosphorylation seems a promising research avenue. Recent evidence also suggests that conflicting selective pressures have shaped arousal patterns. It seems that, when energy stores permit, heterotherms minimize the depth and duration of torpor bouts, perhaps in an attempt to minimize deleterious effects of low T_b.

Potential practical lessons from heterothermy

A detailed investigation of this topic is beyond the scope of this overview, nonetheless I summarize some of the relevant salient points in this section.

Do hibernators resist atrophy?

Over the course of a hibernation season that may last several months, hibernators spend several days completely immobile, punctuated by the brief periods of arousal and IBE. In most mammals, such immobility typically causes significant loss of muscle mass and function, leading many authors to suggest that hibernators are a good natural model for prevention of disuse atrophy. Indeed there are many examples of small hibernators such as bats (174), ground squirrels (111, 203) and prairie dogs (69) that retain muscle mass, fiber size, or protein content throughout the hibernation season. Muscle

function, as assessed by maximal tetanic tension development (174) and dynamic work loops (151) is also preserved in hibernation. Some researchers have suggested that the apparent lack of atrophy is due simply to preservative effects of low T_b (perhaps slowing rates of protein degradation) along with occasional, but intense, activation by shivering during arousal. In golden-mantled ground squirrels, soleus and diaphragm resist atrophy, but some leg muscles (plantaris, gastrocnemius) do atrophy modestly (213). Importantly, this small degree of atrophy is similar whether the hibernators are housed at 4 or 20°C, suggesting that low T_b *per se* has little mitigating effect on atrophy. Supporting this observation, both black and grizzly bears hibernate naturally at fairly high T_b, yet their skeletal muscles resist atrophy, maintaining both fiber size and strength (reviewed in ref. 177). Again these results could be attributed to activation of muscle, perhaps through low-level shivering during the torpor phase. Remarkably, recent data show that grizzly bears are also resistant to denervation atrophy, but only during the hibernation season (177). Taken together these data suggest that, in hibernators, pathways that regulate muscle catabolism are decoupled from fasting, load-bearing, and neuronal activation.

Hibernator atrophy resistance is independent of the presence of muscle-specific stem cells ("satellite cells"; ref. 4). Within gastrocnemius skeletal muscle cells myocyte enhancer factor-2 levels increase, both in the total and active phosphorylated forms, as ground squirrels enter torpor, but return to low levels during arousal (283), suggesting an enhancement of muscle anabolism.

On the catabolism side, increases in muscle concentrations of reactive oxygen species have been linked to proteolysis, atrophy and a reduction in contractile function, but ground squirrel gastrocnemius muscle increases antioxidant capacity more than twofold in the hibernation season (151). Myostatin stimulates muscle catabolism and increases substantially and rapidly in the muscles of nonhibernating mammals following denervation. In 13-lined ground squirrels, however, myostatin and its upstream signals remain low during the torpor phase, but increase during arousal and IBE (35).

Immobility also typically results in bone atrophy, however hibernating ground squirrels retain bone stiffness following 8 months of inactivity better than summer euthermic conspecifics whose activity was restricted within cages (287). This retention of bone function occurs despite a ~20% decrease in bone volume (189). Larger hibernators that experience torpor at low T_b, such as woodchucks (~4 kg), maintain or increase bone microstructure and strength during hibernation (308). Similar results were seen in grizzly bears that hibernate with a T_b near 30°C (190), suggesting that low T_b on its own does not prevent disuse osteoporosis in hibernation. In grizzly bears, this maintenance is achieved despite a decrease in bone turnover (191), presumably by balanced reductions in the activities of both osteoblasts and osteoclasts. From a recent review (80), however, it is evident that the regulation of this balance in hibernation is complex and not yet fully understood.

Are hibernators better at withstanding hypoxia/ischemia?

During hibernation in ground squirrels cardiac output falls 65-fold (231), and blood flow within some organs likely falls close to nil, but is restored during arousal. It is understandably attractive that some authors equate this natural condition with the cold ischemia/warm reperfusion pattern that is common in the clinical use of explanted organs for transplantation. I feel, however, that this is a somewhat oversimplified view, as arousal is a gradual and dynamic process during which cardiac output increases before T_b or MR. Nonetheless protection from ischemia and reperfusion are likely important for hibernators. Moreover, blood oxygen may fall from 120 to 10 mmHg (reviewed in ref. 83), especially during arousal, when hypoxia inducible factor 1α (HIF1-α) accumulates within the forebrain of arctic ground squirrels (182). Despite this apparent hypoxia, 13-lined ground squirrel liver lactate actually decreases during entrance and torpor, compared with summer (253), and even during arousal arctic ground squirrels show no consistent increase in circulating lactate (182), so hypoxia may be intermittent and tissue specific.

The intestine of 13-lined ground squirrel is better able to withstand damage from ischemia and reperfusion than that of the rat, but only during the hibernation season, implying seasonal upregulation of protective mechanisms (166). On the other hand, livers removed from summer ground squirrels and stored cold for up to 72 h retain viability and function better than rats, and this retention of performance actually improves in the hibernation season (178). Accordingly, cardiac arrest has virtually no effect on arctic ground squirrel liver, but causes considerable damage in rats (25). Because mammalian brain is inherently susceptible to interruptions of blood flow and O_2 supply, hibernator brains have been studied extensively. In rats, cardiac arrest damages a large proportion of CA1 hippocampal neurons, but virtually no damage is seen in euthermic arctic ground squirrels (74). Tissue ischemia resistance may also translate to higher levels of organization, as arctic ground squirrels survive extreme blood loss better than rats (25).

Elucidating the mechanisms underlying this resistance is an area of intense current research. Certainly, the suppression of metabolism in torpor would reduce the demand for O_2 and oxidative substrates, and mitochondrial respiration is suppressed in torpor in several tissues (see *Turning Down ATP Production*, earlier), but cold ischemia in rat liver, followed by warm perfusion, does not seem to affect the coupling of liver oxidative phosphorylation (178). Moreover, we found no evidence of mitochondrial metabolic suppression in brain (108), and resistance appears to be independent of maintaining metabolic balance (63). Indeed the inhibition of glycolysis (using iodoacetate) and oxidative phosphorylation (using cyanide) does not affect the survival of ground squirrel hippocampal neurons (63). For brain, a key to such resistance may be the blunting of excitotoxicity that normally occurs in ischemia (reviewed in ref. 171). Macromolecular damage

by reactive oxygen species is also thought to cause damage during reperfusion. During arousal both circulating (285) and brain (220) ascorbate levels decrease with concomitant increases in urate, suggesting increases in ROS production. In arctic ground squirrels, however, this increased ROS production does not appear to cause oxidative damage to proteins or lipids except, perhaps, in BAT (219). These results may suggest an increase in antioxidant capacities during torpor, in anticipation of the increased ROS production during arousal.

Metabolic Flexibility in Ectotherms

Research into metabolic flexibility in endotherms, described in the preceding sections, has many inherent advantages. Owing to potential biomedical applications, funding to study metabolic flexibility in endotherms is somewhat easier to obtain than in other areas of fundamental biological research, and there is an abundance of relevant background information, experimental tools, and models. Without these advantages, research on metabolic flexibility in ectotherms is relatively sparse, but reveals that many vertebrates and invertebrates display metabolic suppression in response to, or in anticipation of, environmental changes. Not only are these research models fascinating, but some offer experimental advantages not available for endotherms. While there are parallels and commonalities with development-linked environmental tolerance, as seen in Dauer larvae of *Caenorhabditis elegans* (301) and embryos of brine shrimp (*Artemia*; ref. 128) and the annual killifish (*Astrofundulus limnaeus*; ref. 230), I will restrict this discussion to metabolic suppression in adult animals.

Definitions and descriptions

By definition, the T_b of an ectotherm is determined by its environment, so it usually tracks T_a. The MR of a typical ectotherm, therefore, declines exponentially as T_a falls (Fig. 1). In regions ranging from temperate to polar, a cold (but suprafreezing) winter can lead ectotherms to become immobile and unable to respond to stimuli as excitable tissues fall below the temperature at which they can depolarize and repolarize (e.g., chill coma in insects; ref. 184). This condition is an inevitable result of passive thermal effects, usually with no other alteration in the animal's physiology or metabolism. Sometimes this condition is referred to as "hibernation," but I believe this term is best reserved for endotherms that show significant and prolonged metabolic suppression in the face of winter environmental stress. During the winter, however, some ectotherms exhibit metabolic suppression that exceeds that predicted by the passive thermal effects due to a decreased T_a. In keeping with other authors (e.g., ref. 29) I will use the term "overwintering" to describe this phenomenon.

Many ectotherms induce metabolic suppression in response to shortages of water or food. These shortages are frequently encountered during the summer, so this condition is referred to as estivation. In some animals, estivation can be induced without a change in T_a or T_b, a powerful experimental paradigm because it allows for the analysis of metabolic suppression without the complication of thermal effects.

Overwintering ectotherms

Most enzyme catalyzed reactions display sensitivity to temperature with a Q_{10} (fractional change in rate over 10°C range) around 2.5. In most ectotherms, this temperature-sensitivity translates to higher levels of organization, so that the Q_{10} of whole-animal MR is near 2.5 (see example in Fig. 1). Although this reliance of ectotherm metabolism on temperature is often taken for granted, there are many instructive exceptions. The temperate north Atlantic fish, *Tautogolabrus adspersus*, has whole-animal MR that decreases at the onset of winter with a Q_{10} value exceeding 10 (68). This whole-animal response corresponds to seasonal decreases in tissue protein synthesis that are extremely temperature sensitive (Q_{10} from 6 to 21) during the transition from autumn to winter (176). Among terrestrial ectotherms, the large South American tegu lizard *Tupinambis merianae* shows a substantial drop in standard MR during the winter, most of which is independent of changes in T_a and T_b (196). The wood frog (*Lithobates sylvatica*) is a well-studied vertebrate ectotherm that overwinters. In this species, while cooling from 4 to 1°C the MR has a Q_{10} approaching 8 (256). Such high levels of temperature sensitivity are usually interpreted as "active," regulated metabolic suppression, as opposed to passive thermal effects on enzyme catalyzed reactions. For the wood frog, active metabolic suppression likely helps to conserve metabolic substrates for use as cytoprotectants, and for mating in the early spring, which occurs before their prey (insects), are available. While this overview does not focus on the remarkable freeze tolerance of animals such as the wood frog (see ref. 282 and references therein for more information), it is noteworthy that MR (as reflected by CO_2 production rates) increases sharply as T_b falls below 1°C and as ice formation is initiated (256). These increases in metabolism likely correspond with mobilization of cytoprotectants, and demonstrate how some ectotherms are not simply metabolic slaves to their thermal environment, but can increase MR even in the face of falling temperatures.

In the case of the wood frog, metabolic suppression can occur under fully aerobic conditions (at least in the laboratory; in the wild they overwinter under leaf litter). In some ectotherms, however, winter metabolic suppression is apparent only when combined with hypoxia. For example, toward the polar edge of its range (65°N latitude), the common frog (*Rana temporaria*) passes the winter submerged in ice-covered lakes and ponds. In this environment, the lack of gas exchange with the atmosphere, reduced photosynthesis due to low light penetration, and continued microbial degradation of organic material lead to progressive hypoxia as the winter advances. Under similar progressive hypoxia in the laboratory, MR (oxygen exchanged across the skin of submerged frogs) declines more than in frogs maintained at cold temperatures but in air-saturated water (82). While anaerobic

metabolism is recruited to some degree in hypoxia, the active suppression of MR is likely key for allowing the frogs to survive this environmental challenge.

Perhaps the vertebrate champion of overwintering is the Western painted turtle (*Chrysemys picta*). Under aerobic conditions the Q_{10} of MR is as high as 8.5 between 10 and 3°C (reviewed in ref. 150). Similar to the common frog, this reptile may pass the winter submerged in ice-covered fresh water, but has a limited capacity to exchange oxygen across its skin. In these conditions, the MR, estimated from calorimetry or lactate accumulation, is ten-fold lower than the aerobic rate at the same temperature (150). These results show that some ectotherms can downregulate metabolism to improve chances of surviving the combination of hypoxia and low temperature.

Despite the reduction of MR, the overwintering common frog maintains metabolic balance within its tissues; after 2 months of hypoxia at 3°C, adenylate energy charge of skeletal and cardiac muscle does not differ from air-breathing controls (82). While enhanced affinity of mitochondria for O_2 might help to maintain ATP supply in hypoxia (263), overall these results suggest that demand for energy is suppressed to the same degree as the capacity to produce ATP. This reversible metabolic suppression is reflected in explanted sartorius muscle (304) suggesting that the effect is intrinsic to the tissue and does not depend on central signals, making it a very useful experimental model. At the cellular level, the permeability of frog muscle to Na^+ and K^+ decreases during cold hypoxia (81). This decrease in ion permeability corresponds with a decrease in the activity and, presumably, ATP demand of Na^+/K^+ ATPase (81). At the mitochondrial level, the leak of protons across the inner membrane of skeletal muscle mitochondria, isolated from frogs overwintering under hypoxia, decreases by 50% compared with those from frogs submerged in normoxic water at the same temperature (262), enhancing energy savings.

As with the common frog, the remarkable anoxia tolerance of the Western painted turtles is reflected in tissues even after removal from the body, and appears to be an intrinsic tissue property. Hepatocytes isolated from normoxic turtles tolerate anoxia at 4°C for many hours with no loss of viability or change in high-energy phosphates (50). Although this tolerance depends on glycolysis (50), anoxia induces a reversible 76% suppression of hepatocyte MR (49). The demand for ATP is reduced, in part by a 75% reduction in Na^+/K^+ ATPase activity under anoxia, though membrane potentials are maintained (48). Profound suppression of protein synthesis (168) and proteolysis (169) also contribute to the maintenance of energy balance. Decreases in the liver and muscle activity of Ca^{2+} ATPase in anoxia likely also contribute to energy savings (235).

In mammals, the brain is considered to be an "obligate aerobe," so it is remarkable to find that the brain of the western painted turtle is as tolerant of hypoxia as its liver. A key to maintaining metabolic balance lies in decreasing the need to pump ions by suppressing neuronal excitability. In mammalian brain, anoxia quickly leads to increased release of the excitatory neurotransmitter glutamate, which activates inotropic receptors, primarily NMDA (*N*-methyl-D-aspartate) and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors. This activation triggers increases in intracellular Ca^{2+}, ultimately causing cell death. In turtle brain, however, anoxia leads to no increase in glutamate, but current and ion flow through NMDA and AMPA receptors actually decrease by 50%, decreasing excitability in a process termed "spike arrest" (reviewed in ref. 46). Like the liver, the anoxia tolerance of the turtle brain is intrinsic, and does not depend on anoxic preconditioning of the whole animal, making it very tractable research model. As a result, understanding the mechanisms underlying anoxia tolerance and spike arrest is an active area of research. These mechanisms are complex and not fully understood, so I will only mention briefly that they likely involve mitochondrial ATP-sensitive K^+ channels, activation of postsynaptic gamma-aminobutyric acid A (GABA-A) receptors (reviewed in ref. 46) and, perhaps, reactive oxygen species (84). A similar sort of "channel arrest" appears to occur in the overwintering pulmonate snail *Helix pomatia*, though the underlying mechanisms remain unclear (161).

Estivating ectotherms

Whole-animal metabolic suppression can be elicited in some ectotherms under benign conditions of oxygen and temperature simply by removing food and/or water. For example, husbandry of garden snails (*Helix aspersa*) is fairly simple, but gets even simpler when food and water are withdrawn at room temperature. The animals retreat into their shell and develop an epiphragm over the operculum, presumably to limit loss of water vapor from gas exchange surfaces. Reducing MR, and thereby the requirement of oxygen further decreases water vapor loss, prolonging the ability to withstand food and water shortage. Within 11 days of withdrawing food and water, with no change in T_a, the oxygen consumption of the snail decreases by 84% (223). Other environmental cues may also be important for triggering estivation. For example in another pulmonate mollusk, (*Oreohelix sp.*) a metabolic suppression of ~80% can be induced within 36 h of introduction of dry air, and is fully reversible when water-vapor saturated air is reintroduced (237).

Lungfish from Africa, Australia, and South America burrow under the surface of the substratum as their ephemeral freshwater habitats dry up during seasonal droughts. They then synthesize a mucous cocoon and breathe only air through a small opening leading to the surface, surviving several months and, perhaps, years until the rains return. This estivation can be induced under laboratory conditions and the MR in an African lungfish, *Protopterus aethiopicus*, decreases by approximately 80%, though full manifestation of this metabolic suppression requires several weeks (96).

Several amphibians inhabiting seasonally dry environments are thought to estivate (228), but thorough characterization of the metabolic responses are wanting for many

species. Removing food and water, but retaining T_a at 24°C, induces a metabolic suppression of over 80% in the green striped burrowing frog (*Cyclorana alboguttata*), allowing it to survive several years of drought burrowed under the substratum in its native Australia (158). On the other hand, in the South American ornate horned frog (*Ceratophrys ornata*) up to 56 days of food deprivation resulted in "estivation," described as no noticeable movement, but with no change in standard MR (124). This example shows us not only that the response among amphibians is not universal, but that functional, metabolic definitions of estivation are more useful than subjective behavioral observations.

Reptiles clearly display seasonal activity cycles, and early reports suggested abilities for metabolic suppression at high, but not low temperatures (199). Since then reports of actively regulated suppression of standard metabolism in response to water and food deprivation are rare and varied (reviewed in ref. 62). In fact, though Australian freshwater crocodiles (*Crocodylus johnstoni*) can survive for several months without access to water they "do not appear to have any specific adaptations for estivation" (61). The superbly named Northern death adder (*Acanthophis praelongus*), also from Australia, experiences a seemingly spectacular 94% decline in field MR during the dry season when prey abundance decreases sharply (160). Most of this decline, however, is due to decreases in activity and digestion, similar to the response of the Burmese python (*Python bivittatus*) to intermittent feeding (250).

In many estivators, the metabolic suppression at the whole-animal level translates to the tissue level. In the striped burrowing frogs, the rate of oxygen consumption by liver and skeletal muscle slices decreases by 50% and 30% when tissue are sampled from estivating animals compared with controls (158). While the preparation of tissue slices is quite simple, it offers little insight into the nature of metabolic suppression. The isolation of individual cells, however, can be a powerful tool for understanding mechanisms that underlie metabolic suppression. Unfortunately, the procedures for isolating cells is laborious, time consuming and invasive, thereby opening the possibility of reversing any mechanisms that elicited the metabolic suppression *in vivo* (see ref. 269, for example). Despite these potential drawbacks, hepatopancreas cells isolated from estivating garden snails display 50% metabolic suppression compared with controls. As in overwintering turtles, a decrease in the energy expended on ion transport likely contributes to this cellular response. The maximal activity of Na^+/K^+ ATPase decreases during estivation in another pulmonate snail, *Otala lactea* (236). In this species, protein turnover is also maximally downregulated after only 2 days of estivation (234).

As in other models of metabolic suppression, estivation effects at the whole-animal and tissue level are often reflected at the mitochondria, though the responses appear to differ among species and tissues. Changes in metabolic fuel preferences also become evident. Following 60 days of estivation in the slender African lungfish (*Protopterus dolloi*) state 3 respiration of isolated mitochondria (fuelled by succinate) is suppressed in liver but not muscle (106). With pyruvate as a substrate, however, white muscle mitochondrial respiration is suppressed by over 70% in estivation, with no change in liver mitochondria (271). Although changes in organelle function were independent of food deprivation (106), fasting alone does decrease liver cytochrome c oxidase activity (107), so the precise mechanism underlying this mitochondrial metabolic suppression remains unknown.

In estivating green striped burrowing frogs, mitochondria from skeletal muscle show up to 83% suppression of both state 3 and state 4 respiration (158). State 4 respiration is thought to represent the basal leak of protons across the inner mitochondrial membrane (Fig. 2), suggesting that estivators have "less inefficient" mitochondria. This decrease in proton leak is greater than that seen in overwintering common frogs (see *Overwintering Ectotherms*, earlier) whereas mammalian heterotherms typically show no decrease in proton leak during torpor (see *Turning Down ATP Supply*, earlier). The suppression of mitochondrial metabolism is not seen in all tissues, including cardiac muscle of the green-striped burrowing frog (238). This result should be viewed with caution, however, as it was obtained using saponin-permeabilized tissue sections (238). We found recently that the inhibition of state 3 respiration of liver mitochondria isolated from hibernating thirteen lined ground squirrels is eliminated when respiration is measured from saponin-permeabilized liver slices from the same animals, implying that the procedure might reverse mechanisms underlying metabolic suppression (K. Mathers and J. Staples, submitted to *Biol. Open*). In estivating garden snails the decline in cellular metabolism occurs even though the number of mitochondria remains unchanged, but the activity of key mitochondrial oxidative enzymes declines in estivation (22). These enzymatic changes likely contribute to the significant reduction in mitochondrial substrate oxidative capacity, though no change in proton leak is noted in estivation (23).

The cellular signals that may control the metabolic restructuring in estivation have been reviewed recently (275), and include many pathways thought to be important in regulating hibernation and daily torpor. More recently compounds of the endogenous opioid system, similar to those thought to be involved in mammalian hibernation (see *How is Torpor Induced?*, earlier) have been implicated. Administration of DADLE and other opioids inhibit the oxygen consumption of liver and skeletal muscle slices from the green striped burrowing frog, and this effect is stronger in estivating animals (159).

Estivation is thought to be a good model for studying resistance to disuse atrophy because animals such as frogs can spend several months essentially immobile, but when rains return they are apparently instantly capable of activities directly relevant to their fitness. Unlike ectotherm overwintering and mammalian hibernation, estivation offers the advantage that it can occur without the complication of change in T_b allowing researchers to discount temperature as a potential complicating factor in experimental design. Indeed

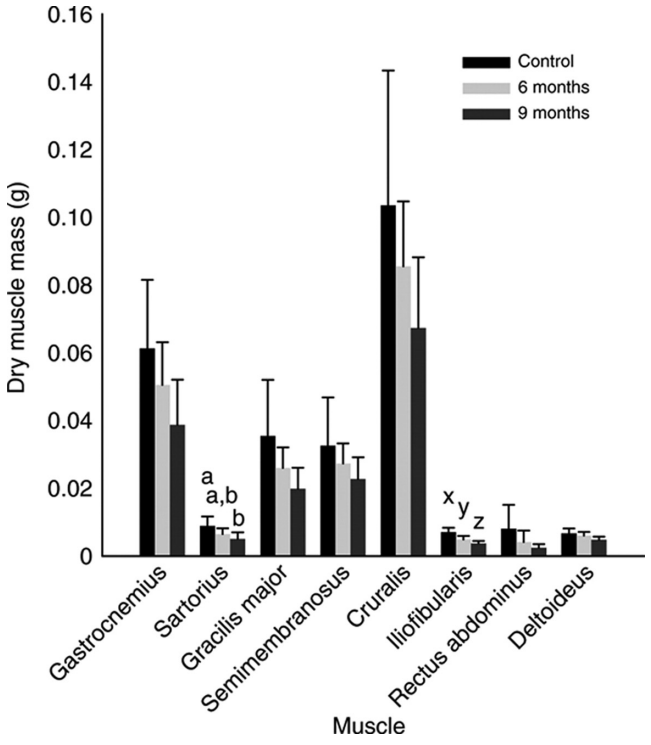

Figure 16 Dry muscle mass (g) of eight skeletal muscles from active and aestivating *Cyclorana alboguttata* ($N = 10$). The letters *a* and *b*, and *x*, *y*, and *z* indicate significant differences. Values are means ± s.e.m. Taken from ref. 186 with permission.

evidence from the green striped burrowing frog shows that 6 to 9 months of estivation produces significant atrophy in muscles that are not employed primarily in jumping locomotion, but little atrophy in important jumping muscles (Fig. 16; ref. 186). This differential pattern of atrophy occurs independently of elevations in temperature and, presumably, tissue MR (312), but does appear to be related to antioxidant capacity (311).

Compared with endotherms, research on metabolic suppression in ectotherms is considerably simpler and offers many experimental advantages. Without thermogenic metabolism assessing potential mechanisms underlying metabolic suppression is more straightforward in overwintering ectotherms. It is clear that some ectotherms suppress metabolism under winter conditions more than would be predicted by passive thermal effects on enzyme-catalyzed reactions. In estivators, such metabolic suppression is achieved with no change in temperature. This whole-animal metabolic suppression is generally retained in isolated tissues, cells and organelles. In many cases, the metabolic suppression appears to be an intrinsic cellular property as it can be elicited by exposing isolated tissues to environmental challenges even if those tissues are isolated from animals acclimated to benign conditions of temperature, food and water that do not exhibit whole-animal MR suppression. Despite these advantages, data describing the extent of MR suppression and the mechanisms underlying it are scant for overwintering and estivating ectotherms, but illustrate some interesting similarities and differences to endotherms.

Maintenance of tissue metabolic balance appears to be important in both overwintering and estivation and, as in endotherms, downregulating demand from important pathways of ATP consumption, including ion pumping and protein synthesis, appears to be an important strategy. Unlike torpid endotherms, however, ectotherms appear to reduce mitochondrial proton leak to conserve metabolic energy. Similar to endotherms, estivating frogs may be a good model for understanding resistance to muscle atrophy.

Conclusion

Every 4 years, the American Physiological Society hosts an intersociety meeting that focuses on integrative and comparative physiology. In 2014, the theme of this meeting was "Comparative Approaches to Grand Challenges in Physiology." This theme fits very well with the current state of research on metabolic flexibility reviewed here. Temporal heterotherms and ectotherms that estivate and overwinter are fascinating models demonstrating the ability to survive under extreme environmental conditions. They may also serve as powerful natural models of tolerance to many important biomedical conditions including hypoxia, ischemia, obesity, and disuse atrophy. Moreover, understanding the mechanisms underlying metabolic suppression may allow for novel treatments of traumatic injury or improvement in organ transplant procedures.

In recent years, there has been a marked improvement in the quality and scope of research in this area. Research quality has been enhanced by improved technologies that allow us to collect more and better data, but I believe the biggest improvement in the field has come from the improved ability to interpret these data and relate them to different levels of organization. As a result "top down," often untargeted genomic/proteomic/metabolomic studies have been able to inform "bottom up" hypothesis-driven, mechanistic studies better than ever, and vice versa. This result comes partly from improvements in bioinformatic analysis techniques, but mostly from improved experimental design originating from researchers who have carefully examined the literature and understand the important factors of the phenomena they study. While this improvement may simply reflect the natural progress of scientific research, it is certainly accelerated by events such as these APS intersociety meetings where both fundamental and applied researchers come together to explore these natural phenomena and their implications. In short, the more we communicate as a group, the better we will be able to design good experiments.

As noted by Hochachka and Somero 30 years ago (145), the best strategy for surviving environmental conditions that may constrain energy transformation is to decrease the demand for that energy. Despite broad phylogenetic distribution, there are many similarities in the metabolic suppression

displayed by hibernators, daily heterotherms, and ectotherms that overwinter or estivate. Perhaps more interesting and informative are the differences, for example, control of hibernation in obligate versus facultative hibernators. From a metabolic standpoint, another fascinating difference is the intrinsic metabolic suppression induced by hypoxia in tissues sampled from normoxic, "normometabolic" turtles, which contrasts with many other models where tissue level responses are seen only when they are examined in hypometabolic animals. Comparing and contrasting such models holds great promise for elucidating the mechanisms underlying these natural phenomena. Integrating data from top-down and bottom-up approaches will accelerate advances in this field. Understanding the significance of changes in enzyme and organelle function, for example, to metabolic suppression will inform targeted searches for intracellular and extracellular signals that initiate the transformations. Moreover, identifying the nature of these modifications and signals may help focus interpretation of the large data sets generated by genomic, proteomic and metabolomics studies. Such an integrative approach can only be achieved through continued improvements in communication and collaboration among research groups.

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