# Coping with Thermal Challenges: Physiological Adaptations to Environmental Temperatures

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## ABSTRACT

Temperature profoundly influences physiological responses in animals, primarily due to the effects on biochemical reaction rates. Since physiological responses are often exemplified by their rate dependency (e.g., rate of blood flow, rate of metabolism, rate of heat production, and rate of ion pumping), the study of temperature adaptations has a long history in comparative and evolutionary physiology. Animals may either defend a fairly constant temperature by recruiting biochemical mechanisms of heat production and utilizing physiological responses geared toward modifying heat loss and heat gain from the environment, or utilize biochemical modifications to allow for physiological adjustments to temperature. Biochemical adaptations to temperature involve alterations in protein structure that compromise the effects of increased temperatures on improving catalytic enzyme function with the detrimental influences of higher temperature on protein stability. Temperature has acted to shape the responses of animal proteins in manners that generally preserve turnover rates at animals' normal, or optimal, body temperatures. Physiological responses to cold and warmth differ depending on whether animals maintain elevated body temperatures (endothermic) or exhibit minimal internal heat production (ectothermic). In both cases, however, these mechanisms involve regulated neural and hormonal over heat flow to the body or heat flow within the body. Examples of biochemical responses to temperature in endotherms involve metabolic uncoupling mechanisms that decrease metabolic efficiency with the outcome of producing heat, whereas ectothermic adaptations to temperature are best exemplified by the numerous mechanisms that allow for the tolerance or avoidance of ice crystal formation at temperatures below 0°C.  *Compr Physiol* 2:2151-2202, 2012.

## Introduction

Animals respond to temperature in a multitude of ways, and over multiple time scales. The actual physiological responses to temperature vary depending on whether body temperature (often abbreviated as $T_b$) is maintained or allowed to covary with environmental temperature. Temperature is a measure of the heat energy present in a system, and as temperature increases, so does the kinetic energy present in the mixture of molecules being measured. At relatively cold temperatures, molecules contain less thermal kinetic energy and collisions between or among them tend to be less energetic ("tend to be" because a single temperature represents only the average kinetic energy possessed by molecules in the system). At warmer temperatures, individual molecules possess more kinetic energy, and collisions and intramolecular oscillations on average are more forceful. These thermodynamic considerations are inescapable, and in biological systems they have profound impacts on the ability of macromolecules, biochemical networks, and cells themselves to function appropriately.

The maintenance of function in the face of changing temperature can be achieved in two fundamental ways: (1) either by adopting and evolving specialized molecular and cellular machinery that bestows tolerance to a wide range of temperatures or (2) by adopting and evolving mechanisms (typically physiological) that allow animals to inhabit varied thermal habitats while keeping an internal temperature substantially different from the prevailing environment. Indeed, the thermal biology of animals is defined primarily by the effects of temperature on biochemical, physiological, and behavioral functions, as well as the strategies animals employ to deal with temperature fluctuations or utilize to assist in maintaining relative constancy in $T_b$. It is, perhaps, safe to say that the maintenance of body temperature mitigates many

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specific adaptations that exist to cope with changing temperatures. Since even simple worms and single-celled organisms will exhibit thermotactic responses to temperature gradients (4,351), thereby effectively exhibiting both an ability to sense and prefer a given temperature, the history of how temperature has shaped the physiological responses of animals dates back to the origins of multicellular animal life.

In this review, we discuss some common thermal adaptations and physiological responses to temperature in animals. We begin with a brief introduction to the maintenance of $T_b$, the varying thermoregulatory strategies that exist within animals, the molecular constraints that drive many temperature sensitive processes, as well as the common molecular, biochemical, and cellular responses that exist, primarily within ectotherms, for countering the thermodynamic influences on biochemical reaction rates. We then consider the genetic basis for thermal acclimation, acclimatization, and adaptation to different temperatures, before discussing specific examples of well-studied responses to cold and heat that exist within ectotherms and endotherms. A comprehensive coverage of all thermal "adaptations" in all animal taxa is not possible here. For example, we do not cover temperature effects on specific physiological systems like the digestive, cardiovascular, respiratory, and locomotory systems (an introduction to these topics can be found in references 150, 175, and 465), nor do we address the evolutionary theories underlying thermal adaptations or whether selection or "phylogenetic inertia" is the driving force for a particular physiological response to temperature (see reference 7). Instead, we focus primarily on the proximate responses that are geared toward compensating for temperature effects on metabolic processes, or for alleviating the effects of changing environmental temperatures.

## Thermoregulatory Definitions

To discuss thermal adaptations, two issues require defining and clarifying. (1) From where do animals derive the majority of their body heat, and how precisely do they maintain $T_b$? (2) For the purposes of this review, we will discuss animals with reference to the following definitions (see Fig. 1):

*Thermoregulation*. The process whereby animals maintain $T_b$ within a restricted range utilizing autonomic control mechanisms that evoke biochemical, physiological, and behavioral processes that modify heat loads internally and externally.

*Ectotherms*. Animals that derive their body heat primarily from the environment (e.g., most invertebrates, fish, amphibians, and reptiles).

*Endotherms*. Animals that derive their body heat primarily from metabolism, from both an elevated rate of resting metabolism and from thermogenic processes (e.g., most birds and mammals).

*Poikilotherms*. Animals that exhibit $T_b$ that tracks environmental temperatures (note: this terminology has fallen out of use, but is often interchangeable with ectotherms).

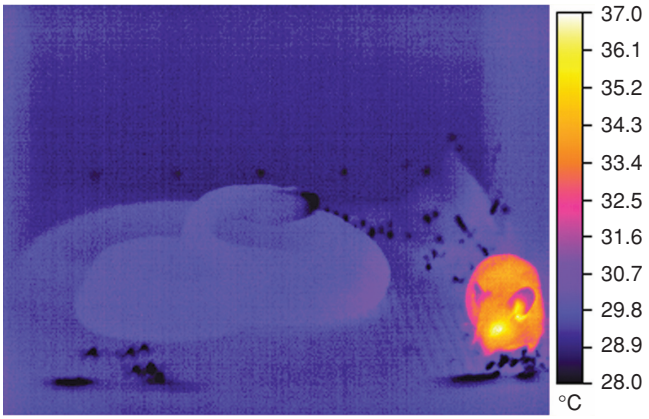


**Figure 1** Thermal image depicting an ectotherm (South American rattlesnake, *Crotalus durissus*) and an endotherm (mouse, *Mus musculus*). The ambient temperature is 30°C. (Images courtesy G.J. Tattersall.)

*Homeotherms*. Animals that maintain a narrow range of $T_b$, across a wide range of ambient temperatures, through the use of homeostatic, primarily physiological mechanisms.

*Heterothermy*. Animals that experience periods of homeothermy, but make use of periodic or spatial variation in $T_b$, typically as an energy saving strategy (e.g., hibernators).

*Acclimation and Acclimatization*. Acclimation is defined as the response to a single experimental variable such as temperature, whereas acclimatization refers to changes in response to naturally occurring environmental changes; for example, seasonal changes, that may comprise concurrent changes in several environmental parameters such as temperature and photoperiod.

## Thermal Sensitivity of Biochemical and Physiological Processes

### Arrhenius dependency and $Q_{10}$ effects

Temperature affects chemical reaction rate, through the laws of thermodynamics. Because the rate of a reaction depends on molecules colliding together (or with an enzyme) and temperature is a measure of the random kinetic motion of molecules, the likelihood of molecules colliding with sufficient energy to react is proportional to the temperature. The kinetic energy of molecules is determined by the absolute temperature (K); $E = {}^1/_2\ mv^2 = 1.5\ kT$, where $E$ is the mean kinetic energy ($J{\cdot}mole^{-1}$), m is mass ($7.78\ 10^{-26}$ $kg{\cdot}mole^{-1}$ for air), v is velocity ($m{\cdot}s^{-1}$), k is Boltzmann's constant ($1.381\ 10^{-23}$ $J{\cdot}mole^{-1}{\cdot}K^{-1}$), and T is absolute temperature (K).

Reactions occur at different rates, and may be influenced by temperature differently. Physical processes rely primarily on molecular collisions, and are only slightly affected by molecular kinetic energy. For example, diffusion rate is proportional to RT, where R is the gas constant (e.g., reference 601). A change in temperature from 293 to 303 K (20-30°C) represents a relatively small change in the average kinetic energy of molecules, about 3% (10/293), hence a small change in diffusion rate. Chemical reactions, in contrast, often have

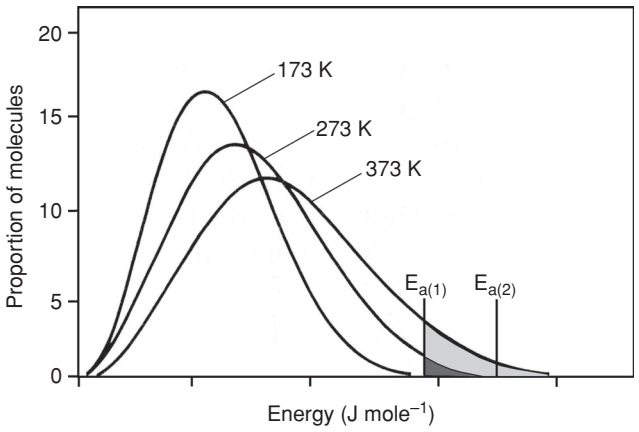


**Figure 2** Schematic representation of the Maxwell-Boltzmann distribution (601) for the kinetic energy of molecules, and the effect of temperature on the number of molecules (gray shading) that exceed low and high activation energies ($E_a$).

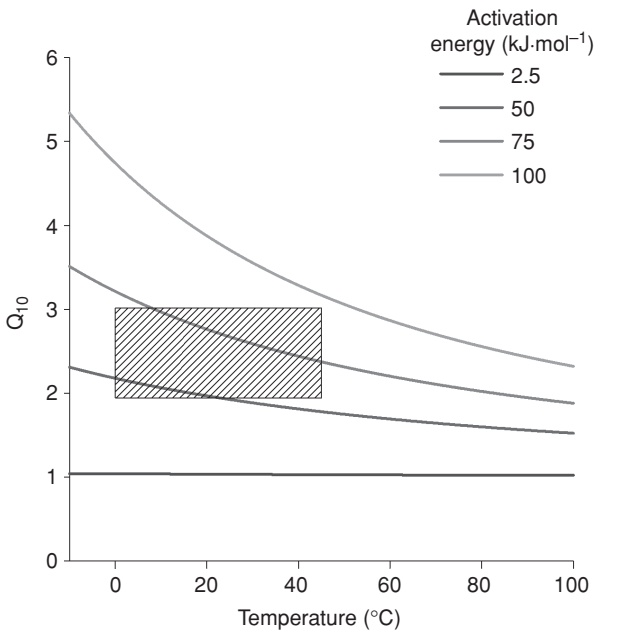


**Figure 3** Influence of temperature on $Q_{10}$ values for processes of different activation energies, ranging from 2.5 kJ·mol$^{-1}$ (diffusion) to 100 kJ·mol$^{-1}$. Hatched area represents "typical" $Q_{10}$ values (between 2 and 3, usually with $E_a$ ~50-75 kJ·mol$^{-1}$) for enzymes at physiological body temperatures.

a much greater thermal dependence because, as recognized by Svante Arrhenius (1856-1927), it is not the mean energy of molecules that influences a reaction rate but the energy content of the most energetic molecules, and a reaction proceeds when a given molecule exceeds $E_a$, the activation energy. When T increases, not only does the mean energy increase, but the energy distribution becomes more right shifted (Fig. 2), following a Maxwell-Boltzmann distribution function; the fraction of molecules with energy exceeding $E_a$ ($E > E_a$) is indicated by the area under the energy distribution. This fraction increases markedly with even a small increase in temperature (cf. $E_a(1)$ and $E_a(2)$ in Fig. 2). Consequently, chemical reactions generally have a higher thermal sensitivity than physical processes such as diffusion. Physiological functions, such as heart rate, ventilation rate, and metabolic rate, being the complicated integral of many physical and chemical reactions, also have a high thermal sensitivity.

One measure of the thermal sensitivity of a reaction is the change in reaction rate (k) resulting from an increase in temperature (from $T_1$ to $T_2$). The Arrhenius equation describes this relationship between rates at two different temperatures, $k_1$ at $T_1$ and $k_2$ at $T_2$; $k_2/k_1 = e^{(E^a/R)(1/T^1 - 1/T^2)}$. This formula indicates a simple way to calculate the $E_a$ for a reaction; the slope for the regression of the natural log of k against $1/T$ ($K^{-1}$) is equal to $-E_a/R$, hence $E_a = -$ slope.R. The $E_a$ for diffusion is about 2.5 kJ·mole$^{-1}$, whereas it is about 50 to 90 kJ·mole$^{-1}$ for many biochemical enzymatic reactions (e.g., pyruvate kinase and cytochrome reductase) and physiological functions (e.g., gill ventilation rate, heart rate, and oxygen consumption rate; see reference 601).

The concept of activation energy is generally used by biochemists to describe the thermal sensitivity of biochemical reactions because there is a specific $E_a$ for a particular chemical reaction (247). For complex physiological processes, the thermal sensitivity can be measured in the same fashion, by regressing the ln (rate function) against $1/T$. The term critical thermal increment ($\mu$) is often used instead of $E_a$. However, physiologists generally do not measure the thermal dependence of complex functions, such as metabolic rate, heart rate, or ventilation rate, by the overall $E_a$ or $\mu$, but by the simple ratio of $k_2/k_1$, standardized to a temperature difference of 10°C (Fig. 3). This ratio is called the $Q_{10}$, where $Q_{10} = k_{(T+10)}/k_{(T)}$. The observed temperature difference is often not exactly 10°C, so $Q_{10}$ can be calculated more generally as $Q_{10} = (k_2/k_1)^{10/(T^2 - T^1)}$. The $Q_{10}$ for physical processes such as diffusion is about 1.03, but for most biochemical reactions and physiological functions it is typically between 2 and 3 (e.g., reference 601). The continued use of $Q_{10}$ as a descriptor of thermal sensitivities is somewhat an anachronism from when it was used as a simple predictor of how biochemical reaction rates change with temperature. Nevertheless, it does provide a useful means to compare different processes' thermal sensitivities, and pervades the thermal physiology literature. There is clearly an equivalence of thermal sensitivity measured as $Q_{10}$ and $E_a$, since they both reflect $k_2/k_1$ for a thermal difference ($T_2 - T_1$). We can calculate that $Q_{10} = e^{(E^a/R)((1/T) - (1/T+10))}$; for a given $E_a$, $Q_{10}$ varies depending on T. For diffusion, at T = 20°C, $Q_{10} = 1.03$, and $E_a = 2.5$ kJ mole$^{-1}$; for many biochemical reactions and physiological functions, a $Q_{10}$ of 2.5 at 20°C corresponds to an $E_a$ of about 70 kJ mole$^{-1}$.

At any particular temperature the fraction of molecules that exceeds the $E_a$ increases as $E_a$ decreases (Fig. 2), hence the reaction rate is increased. Catalysts reduce $E_a$ for reactions and thereby increase the reaction rate. In biological systems, the catalysts are enzymes. These enzymes are complex catalytic proteins that reduce the activation energy required by a reaction and increase the velocity of reactions by many orders

of magnitude. The thermal sensitivity of enzymes adds an extra level of complexity to the thermal sensitivity of chemical reactions and physiological functions.

The capacity of enzymes to increase reaction rates by reducing the $E_a$ depends on a very specific three-dimensional structure of the enzyme's active binding site for the substrate(s) of the reaction (247), and in general this three-dimensional structure is highly conserved amongst different forms of the enzyme (e.g., in different species). It is unlikely that thermal activation of enzymes is a consequence of thermal effects on the three-dimensional active site. In fact, the substrate affinity of enzymes typically decreases at high temperatures (see Section " Implications for alterations in thermal sensitivity"). Rather, the thermal activation of enzymes more likely reflects conformational changes in other parts of the enzyme that are required for the reaction to proceed, and the rates of these conformational changes are likely much slower (hence rate limiting) compared to the speed of actual catalytic conversion of substrates to products. A more flexible enzyme structure could allow more rapid conformational changes, hence more rapid reactions, but a flexible enzyme structure becomes detrimental at high temperatures to substrate binding, the essential catalytic role of enzymes. Thus, there is a trade-off between increased reaction rates at elevated temperatures due to faster conformational changes of the enzyme ($Q_{10}$ = 2-3), with decreased catalytic capacity (higher $K_m$) at higher temperatures ($Q_{10}$ = 0.5-0.9). Proteins denature at very high temperatures, with a rapid and irreversible change in the three-dimensional structure, resulting in an ultimate upper temperature limit for enzyme function. The $Q_{10}$ for protein coagulation is very high (e.g., 30-1000) at these high temperatures, so the $Q_{10}$ for protein "survival time" is very low (e.g., 0.001-0.03). The combination of these three thermal effects on enzyme structure and function results in a typically ∩−shaped temperature-rate curve (resembling the thermal performance curves of animals, although not necessarily due to the same mechanisms; see later).

The thermal sensitivities of biochemical and physiological functions at the cellular level results in similar thermal sensitivities at the system (e.g., digestion, respiration, and circulation) and whole animal level (e.g., locomotion, feeding, growth, development, reproduction, and survival). Thermal performance curves (260) extend from a critical minimum temperature ($CT_{min}$) to a $CT_{max}$ at which the function stops, with an optimal temperature ($T_{opt}$) where performance is maximal ($P_{max}$; Fig. 4). Performance breadth is the range of temperatures over which an animal performs well, and the entire performance range extends from $CT_{min}$ to $CT_{max}$. Thermal performance curves are not necessarily fixed for organisms because they acclimatize to varying environmental conditions (Fig. 5), and performance varies between organisms adapted to differing thermal environments. For example, the performance curves of many biochemical and physiological functions shift with seasonal temperature changes. A low-temperature specialist has a left-shifted performance curve

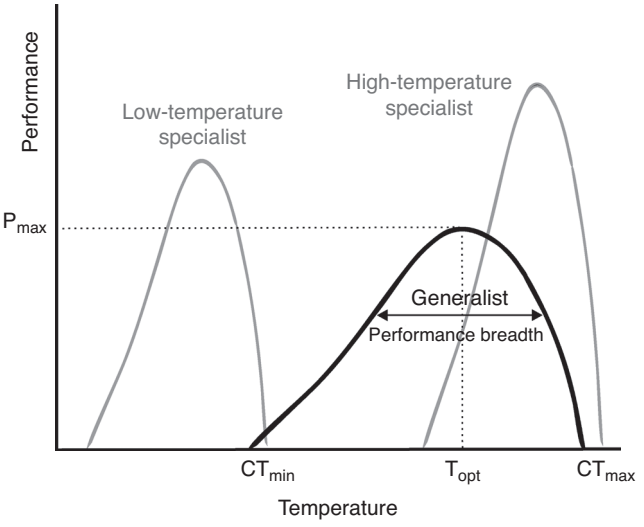


**Figure 4** Schematic representation of a species performance curve (9,260) for a thermal generalist (dark curve), showing the critical thermal minimum ($CT_{min}$), optimal performance temperature ($T_{opt}$), critical thermal maximum ($CT_{max}$) and the performance breadth, and performance curves for low-temperature and high-temperature specialists (light curves).

compared to high-temperature specialist, and both may have a higher maximal performance than more generalist species (Fig. 4).

## Implications for alterations in thermal sensitivity

Given the mathematical relationship between $Q_{10}$ and $E_a$ as expressed previously, it can readily be shown that $Q_{10}$ shows a slight tendency to increase as temperatures decrease (Fig. 3). Although minor, this particular trend does not imply a change

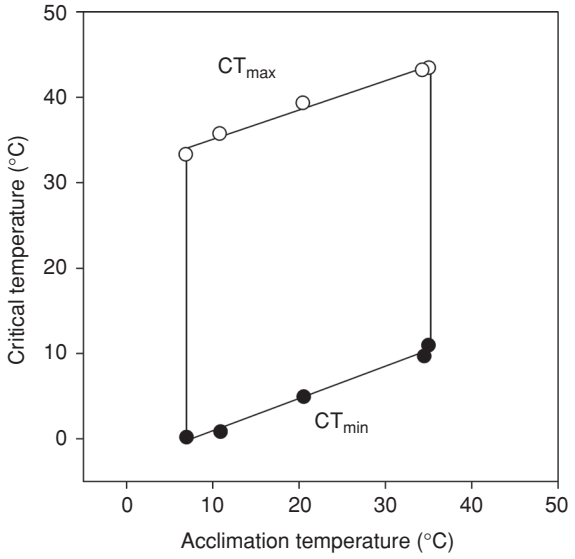


**Figure 5** Critical maximum ($CT_{max}$) and critical minimum ($CT_{min}$) temperatures from stingrays acclimated to a range of temperatures (Adapted, with permission, from reference 171).

in $E_a$, but is rather a natural expectation of the mathematical association. However, there are some precedents for $Q_{10}$ to vary more significantly with temperature beyond this expected relationship. In particular, this phenomenon has been observed numerous times at low temperatures in ectotherms that overwinter (typically below 5°C and above 0°C). $Q_{10}$ values above 4 and ranging up to 10 for whole organism metabolism have been observed in select amphibians and reptiles (57, 162, 534-536, 562), even though $Q_{10}$ values at higher temperatures are more typical (between 2 and 3). This altered thermal sensitivity is often attributed to a change in the $E_a$. Typically, this occurs at an "Arrhenius breakpoint temperature" (ABT), wherein $E_a$ for a particular reaction process is observed to undergo a sudden shift, usually outside of the animal's normal $T_b$ range. For example, ABTs in $Na^+/K^+$-ATPase activity of mammals has been observed to occur at 20°C (447), suggesting a strong suppression of activity at low temperatures, associated with neuropathologies. As a membrane-bound protein, this change in $E_a$ might be due to a phase change in the surrounding lipid environment (632), although not necessarily in all cases. For example, Sokolova et al. (501) examined isolated enzyme activity from marine snails and observed ABTs occurring between 20 and 30°C; these values were also dependent on the study population. Subsequent examination of whole animals demonstrated sudden shifts in the thermal sensitivity curves at temperatures below 10°C (502). They observed $E_a$ approximately 140 $kJ \cdot mol^{-1}$ below the ABT, which was approximately seven times that above the ABT. These changes in thermal sensitivity of metabolic processes have significant consequences for the temperature metabolism curves, leading to a discontinuity in the metabolic relationships; however, the net result is that if these organisms are exposed to temperatures below the ABT, the temperature sensitivity of metabolism is substantially higher, meaning that small fluctuations in temperature will have profound effects on metabolism. The converse is that at elevated temperatures, the metabolic costs of rising temperatures are enormous, since metabolism rises much faster than predicted from typical $Q_{10}$ relationships. Evidence exists, at least in overwintering ectotherms, that they may exploit these extreme thermosensitivities by altering the temperature selection in a manner that bestows substantial energy savings and extends survival time during periods of prolonged energy deficits (440).

Another concern of the exponential relationship between temperature and reaction rates is how fluctuation in temperature influences rate. Since temperature and climate variability (spatial and temporal) are predicted to increase under numerous models of climate change (373), understanding the influence of temperature on physiological responses is critically important to predicting animal responses to climate change. Until recently, the field of thermal physiology has focused more on the static effects of temperature on reaction rates, physiological processes, and animal physiology (504, 568), with only recent interests in temperature variability (for example, see reference 98). However, symmetrical variation (i.e., diurnal) in temperature around a constant mean will not result in a symmetrical variation with respect to rate (due to the Arrhenius equation previously mentioned); this is an example of Jensen's inequality (442). Indeed, it can be shown mathematically that a higher variability around a common mean temperature would lead to an overall increase in the reaction rate of a biochemical or physiological system (599). This particular feature of temperature sensitive reaction rates has implications for the metabolic consequences of climate change. One further consequence of this particular effect is that of predicting climate change effects on physiological responses across the planet. Dillon et al. (148) argue that species from tropical climates will be more affected by thermal variation in terms of metabolic expenditure under climate regimes with increased thermal variation, in spite of the fact that the predicted rise in absolute temperature over the next century will most likely be greater at higher latitudes. Thus, a better understanding of the temperature sensitivities, geographic variation, and possible evolutionary changes that have occurred to modify thermosensitivity would be important avenues for future research.

## Biochemical, Cellular, and Molecular Adaptations to Temperature

### Extreme temperatures

As noted previously, temperature has a powerful effect on the functioning of biological systems, and is one of the most important abiotic environmental factors limiting the distributions of organisms in thermally variable habitats. Such temperature-induced range limitations can be observed across broad latitudinal gradients (192, 503, 523), or across relatively short distances in habitats that have steep thermal gradients such as deep-sea hydrothermal vents (201, 311). Given the clear sensitivity of organisms to temperature, then, the range of temperatures across which life can be found in the biosphere is remarkable. Hot springs famously are host to a diverse assemblage of microbes that can withstand temperatures approaching the boiling point of water (55,60). More impressive, though, are hyperthermophiles isolated from hydrothermal vents, where high hydrostatic pressure allows water to remain liquid at temperatures exceeding 300°C; some of these hyperthermophiles survive temperatures of 121°C (289). These heat-loving organisms, whether from hot springs or vents, are exclusively prokaryotic (432, 455), and there is little evidence that metazoans can survive internal temperatures above 60°C (317), although reports suggest that the hydrothermal vent tube worm *Alvinella pompejana* may transiently survive temperatures of approximately100°C (84) and the moss *Syntrichia caninervis* up to 120°C (517). Some animals (e.g., the midge *Polypedilum vanderplankii*) can withstand very high temperatures while in an anhydrobiotic state, but this high tolerance is not possible when they are physiologically active (246).

At the other end of the thermal spectrum, many organisms, both prokaryotic and eukaryotic, have evolved the ability to tolerate freezing (see Section "Freeze tolerance") and may survive exposure to temperatures well below −100°C (e.g., reference 246), although such tolerance depends on extreme desiccation (43). Active metabolism, in contrast, requires the presence of liquid water, and so these freeze-tolerant organisms survive extreme cold through suspension of metabolic processes. The lowest temperature at which measurable biological activity has been found is approximately -20°C in microbes isolated from polar sea ice and permafrost (132).

Remarkably, there are no novel macromolecules recruited by hyperthermophiles or psychrophiles (cold lovers) to allow biochemical processes to occur at these extreme temperatures; instead hyperthermophiles and psychrophiles make use of the familiar proteins, lipids, and nucleic acids, composed of the same monomeric subunits, as the more familiar mesophiles found in less stressful environments (503). This observation leads to a conundrum—clearly the cellular "machinery" necessary for metabolism, growth and reproduction can be adapted to function across a broad range of temperatures; however, no single species can survive across the full range of temperatures found within the biosphere, and most organisms (i.e., ectotherms) can only function optimally across a few degrees to a few tens of degrees Celsius (247).

What, then, limits the ability of species to occupy wide temperature ranges? For animals, persuasive arguments have been made that in many cases anatomical or physiological limitations lead to suboptimal functioning at temperatures outside the species' normal range. For example, Pörtner and colleagues (420, 421) have convincingly argued that constraints on oxygen delivery to tissues, particularly in aquatic organisms, either due to reductions in oxygen content of body fluids or reduced ability to actively deliver dissolved or bound oxygen, often limit the ability of these organisms to survive extreme temperature fluctuations. However, recent studies in terrestrial arthropods have shown a deviation from this paradigm, suggesting that further examination of the mechanisms of upper and lower thermal limits in animals is required (see reference 524). However, temperature sensitivity of biological systems is universal, and so physiological limitations cannot fully explain the limited temperature ranges of, for example, bacteria, archaea, or plants. There must be additional, universal constraints on optimal function across a wide range of temperatures, and these constraints can be found at the molecular level.

Simply put, changes in temperature affect the structure and stability of macromolecules, and therefore their function. To the extent that the function of any macromolecule, whether lipid, nucleic acid, or protein, depends on its three-dimensional conformation and appropriate interaction with binding partners (substrates, cofactors, transcriptional regulators, etc.), the propensity of temperature change to impact these properties necessarily will modify function of the macromolecule, and thus the metabolic processes of the cell. In this context, proteins have been most thoroughly studied, in part because the relationship between structure and function in this class of macromolecules is well understood. Thus, this description of the effects of temperature, and especially temperature extremes, on biochemical processes will focus on proteins.

## Effects of acute temperature change on protein stability

### *Impact of temperature change on local and global protein stability*

For most proteins, function is absolutely dependent on the maintenance of appropriate three-dimensional structure. The structure of globular proteins can be thought of as hierarchical, with primary structure describing the sequence of amino acids in the polypeptide chain; these "one-dimensional" polypeptide chains fold into a small set of recurring motifs such as α-helices, β-sheets, and loops referred to as secondary structure; tertiary structure then comprises the additional folds that associate secondary structures into larger domains; finally quaternary structure combines separate protein monomers into functionally active oligomers. Importantly, the peptide bond that links individual amino acids into a polypeptide and defines the primary structure of proteins is relatively unaffected by temperature across the range experienced in the biosphere (although see reference 572), and so it is the temperature sensitivity of the secondary, tertiary, and quaternary structural components of proteins—mainly formed and stabilized by noncovalent interactions—that are most important in limiting protein function across broad temperature ranges. These noncovalent interactions, such as hydrogen bonds, hydrophobic interactions, and salt bridges, which are essential to the maintenance of protein three-dimensional structure and therefore function, are described in more detail later.

Catalytically active proteins (i.e., enzymes) must be able to change conformation to function (331, 633). If the challenge of evolving functional proteins consisted simply of creating the most rigid, stable molecules possible, adaptation of proteins to varying temperatures would be straightforward—these inflexible proteins could function appropriately across the temperature range of the entire biosphere. However, in reality, the mode by which catalysis occurs usually involves a modification of protein conformation concomitant with the binding and release of ligands. The conformational shifts occur mainly at the level of secondary and tertiary structure, and require the breaking and formation of some of the noncovalent interactions responsible for maintaining the integrity of the protein at these structural levels. Thus, enzymes have been described as "marginally stable" (142, 271), where stabilizing and destabilizing interactions cancel each other and net stabilization amounts to only a few tens of $kJ \cdot mol^{-1}$, that is, equivalent to a few hydrogen bonds. Thus, changes in temperature can significantly influence the likelihood of local or global denaturation of the protein molecule.

Why cannot enzymes function optimally across a broader range of temperatures? The answer to this question appears to lie in a necessary trade-off between stability and flexibility (490). Optimal function relies on the appropriate binding of enzyme and substrate, which requires maintenance of appropriate three-dimensional structure (i.e., stability), but function also necessarily involves conformational shifts required for catalysis (i.e., flexibility). The resultant marginal stability in many cases limits the temperature range across which proteins can appropriately catalyze metabolic reactions.

### *Impact of temperature change on enzyme—substrate affinity*

Most enzymes are remarkably specific—they can discriminate between substrates with high structural similarity to ensure that metabolic pathways proceed efficiently with minimal waste of material or energy. As first described by Koshland (299), this specificity is based on a combination of three-dimensional complementarity between the protein binding site and the ligand, and the noncovalent interactions that stabilize the enzyme-ligand complex. Based on these considerations, it is not surprising that appropriate binding can only occur when the enzyme active site maintains its native conformation; in other words, a loss of three-dimensional structural integrity of the protein reduces the binding affinity of enzyme for substrate. It is important to note that this loss of structure does not require global denaturation, but instead may be due to increased local flexibility in and around the active site that alters the geometry of amino acids involved in ligand binding. This relationship between stability and binding has been illustrated repeatedly in studies using chemical denaturants (20, 405), pressure (59, 621), or temperature (430, 532) to destabilize protein structure.

A common method to assess the binding affinity of enzyme for substrate is to measure the apparent Michaelis-Menten constant ($K_m^{app}$), which quantifies the substrate concentration necessary for a given amount of enzyme to reach half of maximal reaction velocity ($V_{max}$). An isoform of an enzyme with relatively low affinity for its substrate—that is, one that does not bind substrate strongly—will have a higher $K_m^{app}$ than an isoform with greater affinity. Indeed, studies reporting the relationship between $K_m^{app}$ and assay temperature for enzymes in numerous ectotherms have shown that generally, as temperature increases, the $K_m^{app}$ value also increases, revealing a loss in substrate binding affinity (105, 178, 191, 252, 504). The increase in $K_m^{app}$ with assay temperature takes the form of an exponential curve—whereas at physiological temperatures (the temperature range to which the organism is adapted), $K_m^{app}$ is comparable to the concentration of substrate in the cell, which is usually micromoles to tens of micromoles (1), as temperature increases $K_m^{app}$ also increases so that the amount of substrate necessary to reach half of $V_{max}$ becomes physiologically unrealistic (247). As temperature increases further, $K_m^{app}$ becomes immeasurable, indicating a complete loss of binding, presumably due to denaturation of the protein.

However, it is important to note that at any single measurement temperature $K_m^{app}$ values are not identical among enzyme orthologs from different species, or even among paralogs from the same species. (Orthologs are homologous genes or gene products separated by a speciation event; paralogs are homologous genes separated by gene duplication). When substrate-binding kinetics of orthologous enzymes are compared, the pattern relating $K_m^{app}$ to environmental temperature strongly suggests that binding affinity is adapted to compensate for changes in temperature. Thus, in widely divergent species of ectotherms, and across a variety of enzymes, $K_m^{app}$ assessed at a particular measurement temperature tends to decrease as the temperature range in which the organism has evolved increases (151, 177, 178, 279). However, these studies also show that when measured at physiological temperatures (i.e., within the temperature range to which a particular species is adapted), the $K_m^{app}$ values of differently adapted enzyme orthologs are comparable in magnitude (Fig. 6). Taken together, the temperature sensitivity of enzyme-substrate affinity and the comparable $K_m^{app}$ values among orthologs adapted to different temperatures, when measured at physiological temperatures, indicate that there is temperature compensation in enzyme-substrate affinity.

The fact that enzymes of organisms adapted to differing thermal environments often show compensatory changes in affinity indicates not only that enzyme-substrate affinity is often highly temperature sensitive, but that affinity also can be modified during evolution, and may be under strong selective pressure. In other words, maintaining the appropriate level

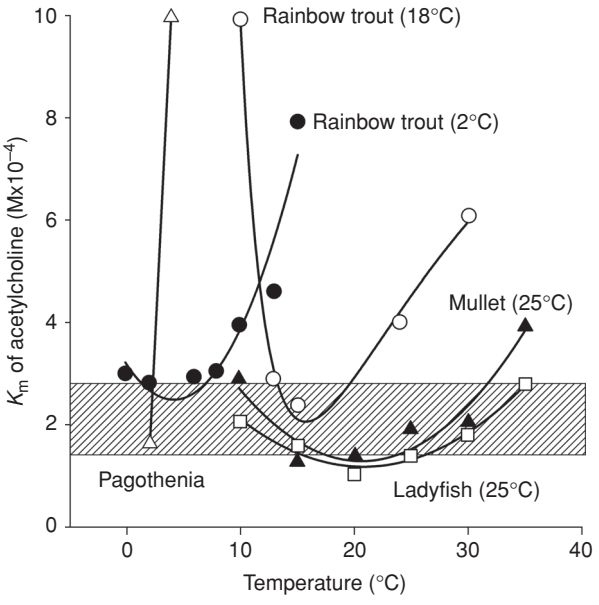


**Figure 6** Effect of temperature on the binding of acetylcholine to acetylecholine esterases of several marine fishes. Shaded area indicates the relatively preserved Km value for the species at their respective habitat temperature. (Adapted, with permission, from reference 27).

of enzyme-substrate affinity appears to be important for optimal metabolic function, and thus an optimal level of stability, necessary to maintain the three-dimensional structure of the active site, must be maintained. The structural changes (i.e., the amino acid substitutions) that can occur during evolution to novel thermal regimes to modify stability and binding affinity are discussed later.

### *Impact of temperature change on catalytic rate*

If substrate affinity was the only parameter that needed to be optimized to ensure appropriate enzyme function, then enzymes could evolve to be as stable as possible, to ensure appropriate binding geometry. Clearly, though, there are countervailing requirements that limit how structurally rigid an active enzyme can be, as evidenced by the rapid rise in $K_m^{app}$ with measurement temperature. The countervailing requirement, in a word, is catalysis. For catalysis to occur, most enzymes must undergo a series of conformational changes, first to bind substrate, then to enclose it within the active site, and finally to release products and return to the initial state (281). The catalytic rate, as measured by turnover number ($k_{cat}$, the number of substrate molecules converted to product per active site per unit time, with units of per second), is strongly affected by temperature, with increasing temperature leading to an increase in $k_{cat}$ (e.g., references 23, 97, 132, and 178). In fact, it is this increase in $k_{cat}$ that underlies $Q_{10}$—the measured increase in rates of biological processes with temperature, often in the range of 2- to 3-fold per 10°C—whether at the molecular or whole-organism level.

The observed increase in $k_{cat}$ of an enzyme is analogous to changes in $K_m^{app}$ described above, and again modifications to enzyme stability appear to be responsible for the altered reaction kinetics (130,400). In the case of catalytic rate, lower temperatures decrease the flexibility of the enzyme molecule, and make the breaking of noncovalent bonds between secondary or tertiary structures within the protein molecule less likely. To the extent that catalysis requires the breaking of these bonds to allow conformational rearrangements necessary for the reaction chemistry to proceed, colder temperatures will retard the process. In contrast, warmer temperatures will enhance the rate at which conformational changes occur, as greater numbers of stabilizing noncovalent interactions break and rearrange, and do so more quickly. Up to a point, then, the warmer the environment is, the faster the reaction will proceed. However, as temperature increases beyond the optimum, too many noncovalent interactions may be broken, so the enzyme molecule loses appropriate structural integrity and cannot bind substrate—that is, localized denaturation can begin (258, 303). Ultimately, at higher temperatures still, the localized unfolding and dissociation of secondary structures will progress to global unfolding and denaturation of the protein, at which point catalytic activity will be eliminated.

As with measured $K_m^{app}$ values, in many enzymes there is strong evidence of temperature compensation among orthologs, such that, when measured at a common temperature, $k_{cat}$ values of enzymes from more cold-adapted species tend to be higher than those from relatively warm-adapted species (e.g., references 23, 177, and 178). When $k_{cat}$ values of orthologs are measured at physiological temperatures, though, these values are comparable in enzymes from species adapted to differing environmental temperatures.

### *A model for adaptation of enzyme function to different temperatures*

The preceding description of the impact of temperature on protein stability, and thus ligand binding and catalytic rate, leads to a conceptual model based on the statistical distribution of enzyme molecules in different conformational/energetic states. This model describes functionally the effects of temperature on enzyme function that is captured quantitatively using the Maxwell-Boltzman distribution described previously. While measurement temperature gives an indication of the kinetic energy possessed by the average enzyme molecule, half of the molecules will have less energy, and so will be relatively stable. Some of these will be in such a low-energy state at any instant that they will not be able to undergo catalytically necessary conformational changes. In contrast, half of the enzyme molecules will have greater thermal energy than average, and some of these may be so energetic that they experience localized, transient unfolding, and so are unable to bind ligand. The $K_m^{app}$ and $k_{cat}$, measured at any temperature, represents an integration of the structural states of all the enzyme molecules in a population. As temperature increases, the average kinetic energy increases, and both $K_m^{app}$ and $k_{cat}$ rise as the population of enzyme molecules becomes more destabilized. To compensate for this effect and keep kinetic parameters within an optional range to maintain metabolic function, increasing temperature necessitates stabilization of protein molecules. Conversely, decreasing temperature requires destabilization.

## Compensatory adaptive and acclimatory mechanisms that alter protein stability

From the previous discussion, it is apparent that enzyme function is significantly affected by temperature change, such that for most enzymes there is a particular range of temperatures in which function—measured by binding affinity and catalytic rate—is optimal. Outside this temperature range, which may extend from a few to a few tens of degrees Celsius, the rate of conversion of substrate to product is not sufficient to maintain adequate metabolic flux, which will negatively influence fitness and survival. The fact that organisms can be found thriving at the extremes of more than 100°C within the biosphere reveals clearly, however, that modifications to protein composition and stability can occur, which allow function to be optimized to any thermal regime in which liquid water can be found. These modifications may be *intrinsic*, that is, they may involve alterations to amino acid composition or location that lead to changes in interactions among secondary

or tertiary structures. Such intrinsic modifications necessarily involve sequence change in the encoding gene, and thus can only occur over evolutionary time. In contrast, *extrinsic* modifications, those that change the number of enzyme molecules present in a cell or modify the surrounding medium itself, can occur more rapidly, and so are useful in acclimatory responses to temperature change (176, 179).

### Intrinsic modifications

As described previously, functional enzymes are only marginally stable, and undergo localized conformational changes as part of the catalytic process. While there may be thousands of stabilizing interactions within a single-folded protein in an aqueous medium, the net stabilization holding the protein in its native, functional conformation may be equivalent to only a few hydrogen bonds (272). This is because there are powerful destabilizing interactions as well. It is the balance between these stabilizing and destabilizing interactions that must be controlled to ensure the protein has sufficient flexibility for catalysis, but retains enough stability to ensure substrate binding.

Entropy is the main thermodynamic parameter responsible for destabilizing folded proteins (200, 324, 356). A typical globular protein, hundreds of amino acids long, can occupy an essentially infinite number of conformations, of which only a small subset would comprise the native (functional) form. Maintaining the native form, then, reduces the disorder in the system, and thus is energetically unfavorable—a denatured protein has greater entropy, and thus the native state must be stabilized by bonds that are enthalpically favorable. Adaptation to temperature change occurs largely by modifying amino acids to alter the number of stabilizing interactions within the protein, or to change the nature of interactions between amino acids and the surrounding medium, such that enzymes adapting to colder temperatures tend to become relatively destabilized (to allow sufficient freedom to alter conformation during catalysis), while enzymes adapting to higher temperatures are relatively stabilized, to ensure denaturation does not occur (400, 430, 548). Examples of the types of structural changes that may occur are described later.

**Amino acid composition** Certain amino acids, as a consequence of their side chain functional group, can stabilize or destabilize protein structures in their immediate environment. For example, glycine is unusual among structural amino acids in that it has no side chain, only a hydrogen atom. The peptide bonds on either side of a glycyl residue can rotate relatively freely, because the lack of a β-carbon reduces steric interactions with neighbors (131, 356). As a result, regions containing glycyl residues have more rotational and translational freedom, and thus greater flexibility. Proline represents a special case of an amino acid that limits rotational freedom. Proline is unique because its side chain attaches to the peptide backbone twice (at the α-carbon and the amino nitrogen) and so eliminates rotation in this location, severely limiting rotational freedom (356). Researchers studying changes in amino acid composition of proteins from organisms adapted to relatively cold or relatively warm temperatures have found an increase in glycine residues in colder orthologs, likely to increase the structural flexibility of these proteins (132, 438), while orthologs from warm-adapted species are relatively enriched in aromatic residues and proline, which may stiffen and thus stabilize protein structure (16, 38, 204).

One caveat regarding studies that examine amino acid composition alone, without describing the location of these substitutions within the protein structure, is that similar substitutions can have profoundly different effects depending location (279, 549). For example, substitutions in the core of the protein likely will have effects unrelated to those on the solvent-exposed surface, and modifications near the active site can affect catalytic processes differently than those located further away. Thus, although changes in the proportions of particular types of amino acids, as described previously, may have effects on protein stability and thus function at different temperatures, the location of these substitutions must also be described to fully understand the mechanism by which protein stability, and thus temperature sensitivity, is modified.

**Hydrophobic interactions** The hydrophobic effect, which is important in stabilizing almost all proteins that function in an aqueous medium (125, 145, 146, 572), arises through unfavorable interactions between nonpolar amino acid side chains within a polypeptide and solvent water (147, 271, 423). Water molecules have the capacity to form hydrogen bonds between themselves or with other polar molecules in solution, and in liquid water these hydrogen bonds break and reform continually as individual water molecules collide and rotate. When a nonpolar solute is introduced to the medium, such as an exposed side chain of a nonpolar amino acid like alanine or valine, surrounding water molecules must accommodate this structure by forming more long-lasting clathrates or cage-like structures, in which water molecules are forced to maintain a particular geometry to allow hydrogen bonding with one another (145, 236). The presence of these clathrates greatly reduces the entropy of the system. As a result, dissolution of nonpolar solutes in solution is unfavorable, and where possible, nonpolar amino acids will interact with one another in the interior of the protein, avoiding contact with the aqueous medium. Put another way, proteins with a substantial hydrophobic core are less likely to unfold, because doing so would force the surrounding solvent to become more ordered.

The strength of the hydrophobic effect increases with temperature (at least within the range of temperatures found in the biosphere), because the energetic penalty of forming ordered, long-lasting clathrate structures increases as water molecules gain more kinetic energy (i.e., heat up). Again, in studies that examine the amino acid composition of proteins from organisms adapted to higher temperatures, the proportion of nonpolar amino acids increases at the expense of polar amino acids (324, 359, 444). In contrast, the hydrophobic effect is weaker at low temperatures—here water molecules have relatively low

rotational or collisional energy and so when nonpolar amino acid residues are exposed upon unfolding the formation of water clathrates does not incur so great an entropic penalty. Thus, proteins that depend on the hydrophobic effect to maintain stability may cold-denature, and cold-adapted proteins tend to have fewer nonpolar amino acids (130, 262, 292).

**Hydrogen bonding and ionic interactions (salt bridges)** In addition to hydrophobic interactions, noncovalent bonds that form between amino acid side chains or atoms of the protein backbone also are important in the stabilization of proteins. Hydrogen bonds most commonly form between N-H and C=O groups of the peptide backbone or polar amino acid side chains (125), which "share" a hydrogen atom and thus structurally link the donor and acceptor atoms. Cold-adapted enzymes, which must undergo catalytically important conformational changes in a medium of low kinetic energy, tend to have fewer hydrogen bonds (133, 573, 617).

Salt bridges occur between ionized residues (positively charged aspartyl and glutamyl residues or negatively charged lysyl and arginyl residues), which interact with partners of opposite charge and thus stabilize neighboring protein structures. Note that, because hydrogen bonds and salt bridges are noncovalent, each bond is relatively weak, but the aggregate of hundreds or thousands of hydrogen bonds and salt bridges within a moderately sized globular protein maintains the native structure while allowing for localized changes in conformation necessary for catalysis (272). Cold-adapted enzymes, which must undergo catalytically important conformational changes in a medium of low kinetic energy, tend to have fewer charged residues (likely indicating a lower number of salt bridges) than do more warm-adapted orthologs (133, 212, 308, 309, 433).

**van der Waals interactions** van der Waals interactions, which also can contribute to stabilization of protein structure, arise when uncharged atoms come in to close proximity with one another. Fluctuations in the position of electrons around the nucleus of one atom create a small, transient dipole that can induce a corresponding dipole in the neighbor. These dipoles attract and stabilize one another, drawing the two atoms together until an optimal distance is reached, beyond which repulsive forces of the orbitals begin to predominate. The strength of van der Waals interactions is highly dependent on the proximity of the constituent atoms (the attractive force decreases with the sixth power of distance; 125), so in folded proteins stabilization by van der Waals interactions is dependent on tight packing of residues (64). It appears that efficient packing of amino acid residues depends on the volume of amino acid side chains, with larger, bulkier side chains packing more tightly in the interior of the protein and thus leading to greater stabilization. Supporting this model of the role of efficient packing and van der Waals forces in temperature adaptation, warm-adapted proteins tend to have proportionally more amino acids with larger, bulkier side chains such as tryptophan and phenylalanine (81, 250, 386, 399), although more efficient packing of nonpolar residues can increase stabilizing hydrophobic interactions, as well (88).

**Disulfide bridges** In addition to the stabilizing noncovalent interactions among amino acids in a polypeptide, protein structure can be stabilized as well by covalent bonds, most notably disulfide bonds between neighboring cysteinyl residues. Based on the strength of covalent relative to noncovalent bonds, one might assume that disulfide bridges are an important part of the evolutionary toolkit for stabilizing proteins against thermal denaturation. In fact, disulfide bonds are uncommon in intracellular proteins (271), and appear only in proteins targeted to the extracellular milieu. As a result, for the vast majority of proteins, disulfide bridges are not used to stabilize structure. This may be because covalent bonds require an input of so much energy to break that they limit the inherent flexibility of enzymes necessary for adequate function. Alternatively, disulfide bridges may not be an available component of stabilization of intracellular proteins because the intracellular environment is kept in a highly reducing state. The ratio of reduced to oxidized glutathione ([GSH]:[GSSG]) in a healthy cell often is greater than 100:1 (451), and so any disulfide bonds formed would very quickly be re-reduced to the thiol groups. This leaves only proteins targeted to the exterior of the cell, where conditions are oxidizing, as candidates for stabilization by disulfide bridges.

**Changes in secondary structure** The previously mentioned mechanisms responsible for altering that stability of protein molecules, and therefore allowing adaptation to temperature, depend on specific types of amino acid substitutions. Proteins may also be relatively stabilized or destabilized by larger scale modifications to secondary structure. For example, many proteins have relatively disordered loop structures that are not stabilized by noncovalent interactions. Evidence suggests that proteins from thermophilic organisms tend to have fewer, shorter loop structures, and are smaller in general than mesophilic or psychrophilic orthologs (106, 558). Researchers have suggested that this reduction in size in thermophilic forms arises because it reduces the difference in entropy between the folded and unfolded state, thus stabilizing the folded state relative to a larger protein (547). Other structural changes that may lead to stabilization of warm-adapted proteins include an increase in the number of β-sheets relative to α-helices (78), and the strengthening of helix dipoles, where the insertion of charged residues into an α-helix in sites that reinforce the existing helix dipole, stabilizing the structure (79, 575).

### *Extrinsic modifications*

The intrinsic or structural modifications to protein stability that are described previously allow adaptation to different thermal environments, but require changes to amino acid composition and thus only occur on evolutionary time scales. In contrast, other modifications extrinsic to protein structure can

occur on relatively short time scales, and so may be useful in acclimation to rapidly changing thermal environments. Two examples of such extrinsic modifications are changes in the copy number or type of isozyme expressed, and alterations in the composition of solutes in the cytosol, which may stabilize or destabilize the native state of the protein.

**Enzyme copy number and isoforms** Acute exposure to cold will necessarily reduce the catalytic rate of most enzymes, as a loss of kinetic energy inhibits the conformational changes necessary to allow catalysis to occur. To maintain metabolic flux in the cold, a straightforward if energetically costly strategy is to synthesize more copies of the enzyme acting as a bottleneck to flow of metabolic intermediates within a particular pathway (235). For example, cytochrome c in green sunfish (489) and both carnitine palmitoyl transferase I and citrate synthase (CS) activity in striped bass (437) increased significantly after cold exposure, likely due to an increase in the number of mitochondria, and therefore proteins. Similarly, in a study of cold acclimation in horse mussels, Lesser and Kruse (320) measured increases in activity of a number of metabolic enzymes including CS and hexokinase, as well as antioxidant enzymes and heat shock proteins, which they interpreted as indicative of increased metabolic flux in response to decreased catalytic rates of enzymes. Although increasing the copy number of enzymes during cold acclimation is straightforward, it is far from universally observed, nor is temperature compensation due to synthesis of additional enzyme molecules always complete (387). This may be because the process of cold acclimation via an increase in enzyme copy number, or in numbers of organelles such as mitochondria, is energetically costly—transcription and translation demand large inputs of ATP and nucleic acid or amino acid building blocks. Nevertheless, increasing enzyme copy number can increase metabolic flux during acute temperature change, and may be preferable to lower metabolic rates that may limit physiological or behavior scope for activity.

An alternative mechanism to modify enzyme catalytic rates in response to temperature change is to express different isoforms when exposed to different thermal environments (631). Although this strategy requires multiple copies of a gene, likely arising through gene duplication, in those organisms that have the luxury, these "extra" gene products may become adapted to function optimally at different temperatures (28). However, because of the need for multiple copies of genes encoding particular enzymes, even in species that show evidence for isoforms adapted to different temperatures, a relatively small proportion of enzymes are affected (477).

**Stabilizing and destabilizing solutes** A second mode of extrinsic modification of enzyme function in response to acute temperature change involves modifying the aqueous milieu in which cytosolic proteins function. Certain "compatible" solutes (618, 619) have the property of stabilizing or destabilizing folded proteins generally, and do so through modifying the nature of protein-solvent interactions. The mechanism by which stabilizing solutes function was elucidated by Timasheff and co-workers (15, 48, 199), who described a process of "preferential exclusion" where solutes are excluded from the volume of solvent water immediately surrounding the protein molecule. These solutes, such as polyethylene glycol, sucrose, trehalose, certain amino acids, and trimethylamine-*N*-oxide (TMAO), among many others, may be excluded from the surface of the protein for a variety of reasons, including size (steric hindrance; 48), increased surface tension (329), or unfavorable interactions with amino acid side chains or the peptide backbone (39). Whatever the immediate cause of the exclusion, however, the effect on protein structure is the same—the existence of a volume of solvent water from which solute is excluded is energetically unfavorable, since it decreases the entropy of the system. In response, the volume of exclusion is minimized, and this occurs through the minimization of the surface area, and therefore volume, of the protein. In other words, preferential exclusion of these compatible solutes leads to stabilization of the protein native state at the expense of denatured conformations with greater surface area. As a result, the synthesis of stabilizing solutes can counteract the destabilizing effects of high temperature, and the use of stabilizing solutes is found in many organisms exposed to rapid, transient spikes in temperature. Numerous studies have described the accumulation of compatible solutes, not only in response to high temperature (173) but also during exposure to other denaturing stresses such as high salinity (449) and high pressure (448). The use of stabilizing osmolytes occurs widely, having been found in taxa including bacteria (393), archaea (355, 390), plants (445), and animals (620), to provide only a few representative examples. It is important to note that the stabilization achieved by these compatible solutes is independent of the type of protein; that is, because of the mechanism by which these solutes act, any aqueous globular protein should be stabilized in their presence. As a result, the synthesis of compatible solutes can act to stabilize the majority of proteins in a cell rapidly, without requiring specific modifications to each temperature-sensitive polypeptide chain.

Although there have been many examples found of compatible osmolytes providing protection against protein denaturation at high temperature (as well as in other destabilizing environments), until recently there has been less evidence for the opposite process—accumulation of chaotropic or denaturing solutes in cold-acclimated organisms, to reduce the stability of enzymes and increase catalytic rate. Chaotropic solutes generally will interact favorably with functional groups of the protein, in contrast to the stabilizing osmolytes, and this favorable interaction will compete against the stabilizing interactions serving to maintain native structure (42). At high concentrations, chaotropes such as urea will completely denature globular proteins, but at lower concentrations, it is possible that localized flexibility may be increased in a manner that allows increases in $k_{cat}$. Chin et al. (85) have shown that psychrophilic fungi not only grow faster on media supplemented with chaotropic solutes but also synthesize and

accumulate chaotropes at low temperature. These data suggest that compatible solutes may be used more widely than was previously suspected—not only in environments where protein denaturation is a danger but also in situations (such as cold conditions) where excessive stability of proteins may reduce metabolic rates.

## Genetic Adaptation and Acclimation to Temperature at the Organism Level

### Thermal signals and response times

Temperature is one the most pervasive physical parameters affecting the fitness of organisms (232). Consequently, the thermal environment exerts strong selection pressures and the interaction between organisms and their thermal environment is a focal research area in physiological ecology and evolutionary biology (7, 267). The effect of thermodynamics on organism function (phenotype) may be modulated by compensatory responses. For example, organisms in cold environments may produce greater quantities of a particular enzyme to either compensate for a temperature-induced decrease in catalytic activity in ectotherms (217) or to increase heat production capacity in endotherms (168). Such responses may occur at different time scales: between generations (genetic adaptation; 172), during development (developmental plasticity; 557) so that phenotypes are matched to prevailing environmental conditions, and reversibly within phenotypes (reversible plasticity; 600). Hence, responses to thermal variation are a composite made up of responses at different temporal scales. At each temporal scale, ranging from hours to many years, there will be a lag between environmental and phenotypic change, and the phenotype will be uncoupled from the environmental signal following a phase change in environmental conditions. The lag period is determined by the capacity of individual traits to respond to environmental change.

The current theory underlying most research in thermal ecology is based on the seminal work by Levins (323). Accordingly, the optimal "adaptive strategy" of organisms would depend on the patchiness or "grain" of the environment. A coarse-grained environment fluctuates between distinct states, and a phenotype is unpredictably exposed to only one. In this case, total fitness will be determined by the product of the individual fitness components for each environmental state. Performance and fitness in a coarse-grained environment may be maximized by genetic adaptation if environmental conditions remain stable across generations, and by developmental plasticity if the environment remains stable during the lifetime of the organism (323,484,588). In a fine-grained environment a phenotype experiences numerous patches, so that total fitness will be the sum of the individual fitness components of each patch, and reversible acclimation would enhance performance and fitness (515, 600). In theory (323), coarse-grained environments will produce phenotypes that are specialized to the relatively stable conditions experienced, while fine-grained environments produce generalists that perform well over a wider range of environmental conditions albeit at a reduced level; in other word, generalists trade-off maximal performance for performance breadth. However, this need not be the case if reversible acclimation can compensate for environmental variation experienced during the lifetime, effectively leading to "specialized generalists" in which the temperatures at which performance optima occur track changing thermal conditions without loss of total performance (202, 463).

Most species experience both fine- and coarse-grained variation at several temporal (e.g., day, season, and geological) and spatial (e.g., microhabitat and latitude) scales, as well as the interaction of the two resulting from animal movement (472). Hence, fine scale patchiness at a short temporal scale (e.g., season) is added to coarse scale variation at longer periods (e.g., climate change). Responses to thermal variation are therefore likely to be a composite, comprising genetic adaptation, developmental plasticity, reversible plasticity, and acute responses such as behavioral selection of microhabitats or rapid changes in heat production. Note that adaptation and plasticity are fundamentally different from acute responses, because the latter operate within a particular thermal sensitivity curve (reaction norm), whereas the former shift the reaction norm along a temperature continuum. Hence, plasticity and adaptation change the capacity of a physiological trait at different temperatures, while acute temperature variation will modulate instantaneous rates (see Fig. 2).

The relative importance of plasticity and adaptation will depend firstly on the relationship between lifespan and rate of environmental change and secondly on the rate of phenotypic change relative to environmental change. The importance of lifespan is that species with very short life spans may experience only one distinct (coarse-grained) environment so that genetic adaptation between generations and possibly developmental plasticity will be the most important responses. However, many species will also experience at least seasonal variation within their lifetime, in which case the optimal adaptive strategy will be a flexible phenotype in which fitness is maximized at each of the predictable thermal extremes in addition to genetic adaptation to geological climate change or to latitudinal gradients (172, 416). Responses to thermal variation may be conceptualized as a Fourier series where several cycles with different frequencies are superimposed on one another, and where total fitness will be determined by the change in mean trait value and the phenotypic plasticity surrounding it.

The potential for phenotypic changes in physiological capacity (as opposed to changes in instantaneous rates) vary between traits and species. Ideally, fitness is maximized when organisms can perform at a constant level despite environmental variability. However, it is impossible that the phenotype can change at the same time as the environment if the environmental change provides the signal for phenotypic change. Hence, there will always be a lag between the two. The lag in the phenotypic response may preclude plasticity when the

rate of environmental change is greater than the potential for phenotypic change. Hence, an environmental fluctuation with a period that is much shorter than the response time could not act as a stimulus for phenotypic change. For example, it will take several weeks for changes in metabolic gene expression and enzyme activity to compensate for a chronic change in temperature (56, 402, 439) so that daily temperature fluctuation will not affect metabolic capacity. Generally, acute changes in temperature resulting from movement through different microclimates, weather changes, and diurnal fluctuations may affect real-time physiological rates, but do not affect capacities. This is advantageous because modulation of physiological capacities can incur costs such as production of reactive oxygen species and ATP use by protein synthesis (217, 465). Similarly, genetic adaptation at an evolutionary time scale will occur only when the rate of environmental change is slower than that of genotypic change so that individuals experience a relatively stable thermal signal between generations.

A critical period for phenotypic change occurs when there is a phase change in the environment, which means that the phenotype changes in opposition to the environment, and the resulting dissociation between environment and phenotype has potentially negative fitness consequences. The severity of the dissociation depends on the lag between environmental and phenotypic rates of change: the greater lag, the longer the period of dissociation. Periods of dissociation arise commonly with seasonal change, but in organisms that acclimate seasonally, the lag is presumably short so that the decrement in fitness is negligible. At an evolutionary time scale, however, a major shift in climate may cause extinctions because the time lag would be large. This may occur particularly after catastrophic climate changes, such as the purported meteorite strike which is suggested to have cause a major climate change and mass extinctions at the K-T boundary (396). Similarly, human induced climate change may cause extinctions if phenotypes are fixed, and genetic adaptation is the only possible response.

## Developmental plasticity

A given genotype can give rise to different offspring phenotypes depending on environmental conditions (417,557,588). Environmental conditions during development can alter offspring phenotypes irreversibly by acting either directly on the offspring (intragenerational effects) or via signals passed on from the parents (intergenerational effects). The mechanisms underlying intragenerational effects of developmental conditions are not clear. In many cases where such effects have been reported, it seems that stressful conditions during development caused irreversible changes in the offspring phenotype (600). Incubation temperatures can influence offspring phenotypes directly (61) but do not necessarily have an effect (6).

In contrast, intergenerational (paternal or maternal) effects are becoming better understood and are currently at the cutting edge of research into the link between phenotype and genotype (283). Parental effects can be mediated by transfer of material (e.g., mitochondria) from the maternal and even paternal gametes to the offspring cells (380). Importantly, both parents can affect offspring phenotypes by altering DNA molecules and thereby influencing offspring gene expression patterns (394,434). DNA molecules can be altered chemically by DNA-methyltransferases that transfer a methyl group from the *S*-adenosyl methionine to the cytosine ring (296). Methylation of DNA results in gene silencing by restricting access of transcriptional regulators, or by direct suppression of methylated cytosine-guanine dinucleotides (51), and it is a potent mechanisms of programming gene expression (288). In mammals, there are several methyltransferases, which either introduce novel cytosine methylation (DNMT3a and DMNT3b) or copy existing methylation patterns to the new DNA strand during DNA replication (DNMT2; 296). If DNA methylation occurs during early development, it may stably alter offspring phenotypes in adult life. Environmental conditions experienced by parents can change methylation patterns of offspring DNA and thereby modulate offspring phenotypes. The advantage of this epigenetic modification is to match offspring phenotypes to prevailing environmental conditions. Maternal nutrition plays a crucial role in methylation patterns of offspring DNA, which alter postnatal behavior and metabolism (40). Hence, sedentary behavior (571) and metabolism (203) of mammals can be determined by prenatal conditions via methylation of DNA. Even variation in postnatal maternal care can influence methylation state of offspring DNA and thereby determine behavior and stress responses later in life (288, 585). Additionally, the mother's nutritional and physiological status, as well as temperature may influence the size and condition of her eggs and fetus (40), which in turn may influence the future growth trajectory and developmental pattern of the offspring (46, 273, 332, 338). These maternal effects may be detrimental if parental influence is in opposite to the environments experienced by the offspring. On the other hand, even seemingly negative influences such as a poor nutritional state of the mother may be beneficial because it can produce an offspring phenotype that is small and has a low metabolic rate, and is therefore better suited to a nutritionally harsh environment.

Epigenetic modifications of DNA can be transmitted across generations (434), and epigenetic processes may have similar effects on offspring phenotypes as genetic adaptation resulting from changes in nucleotide sequence. Transgenerational transmission of epigenetic patterns has the potential to match offspring phenotypes to longer term (relative to lifespan) changes in the environment. Parental epigenetic modifications such as DNA methylation can therefore increase offspring fitness at different temporal scales. Interestingly, DNA methylation is extremely dynamic and methylation patterns can be altered even during the lifetime of an organism, and demethylation and remethylation can occur successively on promoters of at least some genes (50, 366). The significance of these dynamics for thermal biology of animals

remains unresolved. Temperature *per se* can act as a trigger for setting characteristic DNA methylation patterns and thereby determining gene expression in plants (478), and there is a correlation between the body temperature experienced by different species and DNA methylation patterns (63, 269, 565). However, the processes underlying overall genome methylation across species are different (mediated by DNMT1) from the de novo methylation resulting from environmental and parental influences (mediated by DNMT3a and b; 565). The effect of DNA methylation on individual performance and fitness and the importance of parental influences remain unresolved.

## Reversible plasticity

Reversible phenotypic plasticity may be defined as a change in the thermal sensitivity of a performance trait that occurs in response to an environmental change, and which is reversible within an adult organism. Acclimation and acclimatization have been defined earlier; however, for simplicity we will refer to both phenomena as acclimation here. A special case of reversible plasticity is heat hardening, which is mediated by the expression of heat shock proteins and other chaperone proteins following exposure to extreme temperatures (249, 304). Unlike acclimation of physiological capacities, heat hardening occurs at temperature ranges that cause damage to proteins, which is alleviated or at least retarded by the heat hardening response, which may last for several hours.

Acclimation of physiological capacities is beneficial because it may compensate for a potentially negative influence of an environmental change on a fitness-related performance function (217, 600). There has been considerable discussion in the literature whether or not acclimation is beneficial (333), often leading to rejections of the "beneficial acclimation hypothesis" (e.g., reference 319). However, many tests of the benefits of acclimation were confounded by poor experimental design by, for example, failing to isolate reversible plasticity from intergenerational and developmental processes, or by exposing organisms to damaging temperatures (600). Additionally, absence of acclimation in a particular trait may indicate lack of plasticity in that trait or that the trait is not limiting performance or fitness, neither of which permits the conclusion that acclimation is not beneficial.

Acclimation is best known from metabolic responses to temperature change (186). Many species from a broad range of taxa have the capacity completely or partially to compensate the depressing effects of decreasing temperatures on metabolic functions (218, 515, 546). Ultimately, cellular function is maintained by controlling the stoichiometry of biochemical pathways, which means that maximal flux is not necessarily optimal, and acclimation may also be interpreted as decreasing flux in response to temperature increases (465, 546). Although acclimation is best known from metabolism, similar responses occur across a wide range of traits, from transcription to locomotion (223, 282, 318). The importance of acclimation is that the response is rapid; physiological changes are established within days to several weeks. Hence, it is the principal mechanism that permits organisms to maintain fitness in environments that vary within generations.

The enabling mechanisms underlying acclimation are unknown, which makes it difficult to deduct its mode of evolution. Acclimation could be an inherent quality of a cell where energy sensing mechanisms could act to maintain constant flux across environmental gradients. If for example, energy (ATP) consuming and producing pathways have different thermal sensitivities, the relative concentrations of AMP may fluctuate at different temperatures. This would stimulate AMP-activated-protein kinase (AMPK) to adjust metabolic flux according to the cells' needs (224). Most downstream processes such as locomotion and growth rely on an adequate ATP supply so that AMPK activity could explain acclimation beyond metabolic pathways. The AMPK system has evolved very early in the evolution of life, and it is thought to be a necessary biochemical prerequisite for the evolution and function of cells. Hence, it is ubiquitously present in all cellular organisms and could therefore be regarded as an inherent function of a cell.

Alternatively, acclimation may be controlled centrally, which would require the presence of thermal-sensing mechanisms and the facility to process thermal signals leading to an efferent response to tissues and cells (464). The central nervous system of vertebrates has such a capacity with transient receptor potential ion channels as temperature sensors that provide the afferent signal to the hypothalamus, which initiates a sympathetic efferent response. The nervous system of invertebrates may have similar capacities, but cold hardening can be induced in cultured insect cells (625), and if central mechanisms were solely responsible for an acclimation response it would be expected that very simple metazoans and unicellular animals could not acclimate.

Acclimation could also be facilitated by the evolution of particular genes or alleles. For example, isoforms of lactate dehydrogenase possess different thermal sensitivities, and different isoforms are expressed differentially under different thermal conditions, thereby maintaining total flux of the pathway despite environmental variation (503).

These examples of possible enabling mechanisms underlying acclimation are by no means exhaustive, but they demonstrate that acclimation cannot be viewed as a single evolved trait. The evolutionary processes that led to complex nervous systems, for example, are fundamentally different to those underlying the AMPK system or the evolution of single genes. Hence, verbal and mathematical models that attempt to explain the evolution of acclimation as though it were a single evolved trait are of limited utility in explaining the phenomenon. Importantly, acclimation responses may be specific to particular performance traits. For example, acclimation of locomotion may partly depend on underlying metabolic processes but also on muscle specific processes such as myosin ATPase activity; metabolic ATP production may depend on the AMPK system, but at the same time also on the fatty

acid composition of mitochondrial membranes. Hence, the constraints for acclimation are not the same for any two traits. It is unlikely that a single mechanism is responsible for acclimation as a phenomenon; rather, evolutionary changes in several control mechanisms are likely to explain the phenomenon within an organism. Until more is known about underlying enabling mechanisms, discussions about evolutionary processes must be treated as preliminary.

## Physiological Responses to Temperatures

The following sections on low- and high-temperature physiological responses provide specific examples of well-established physiological responses geared toward maintaining specific body temperatures or coping with thermal stresses. A discussion of whether a given response can be referred to as an adaptation in the absence of genetic data or selection experiments is a germane point to the discussion of the literature. Given the historical significance of physiological research in shedding light on the inherent homeostatic mechanisms that animals exhibit in response to fluctuations in their environments, it becomes a primary academic argument to dispute whether a neurological reflex to a change in temperature is or is not an adaptation to changing temperatures, and is best left to theoretical biologists to dispute.

## Low-Temperature Adaptations in Endotherms

Mammals and birds have independently evolved an endothermic strategy of thermoregulation, where body temperature ($T_b$) is maintained relatively constant by proportional metabolic heat production (MHP). At ambient temperatures ($T_a$) below $T_b$ there is a thermal gradient ($\Delta T = T_b - T_a$) between the animal and its environment, and heat is lost from the animal to its surroundings. The greater is $\Delta T$, the greater is the rate of heat loss. Therefore, low temperatures impose significant energetic consequences for endotherms, reflecting the higher MHP required to maintain a constant $T_b$ as $T_a$ declines. For small species in particular, with a high surface area to volume ratio and limited capacity to store energy reserves or accumulate insulation, cold environmental conditions present a substantial physiological challenge. Endothermic mammals and birds use three general strategies to deal with low ambient temperatures and associated increased heat loss; they can increase their rate of heat production or radiative heat gain, they can decrease their rate of heat loss, or they can allow body temperature to drop, reducing $\Delta T$.

### Heat-production mechanisms

Most endotherms are homeothermic, defending a high and relatively constant $T_b$ against a considerable $\Delta T$, with a proportional increase in MHP when ambient temperature decreases (Fig. 7). The increased MHP results from modification of normal energy converting processes to more inefficient states, maximizing the release of heat from chemical energy. There are two main heat-producing processes; shivering, and nonshivering thermogenesis (NST; 165). The principal mechanism for augmented heat production is shivering, the uncoordinated or nonsynchronous contraction of skeletal muscles, at about 10 to 20 contractions per second (482). Antagonistic muscle motor units are activated contemporarily by the nervous system so that opposition of the contractions results in little gross movement, although the conversion of ATP to ADP required to power these contractions produces heat (165). Skeletal muscle is capable of considerable heat generation due to its high aerobic capacity and large

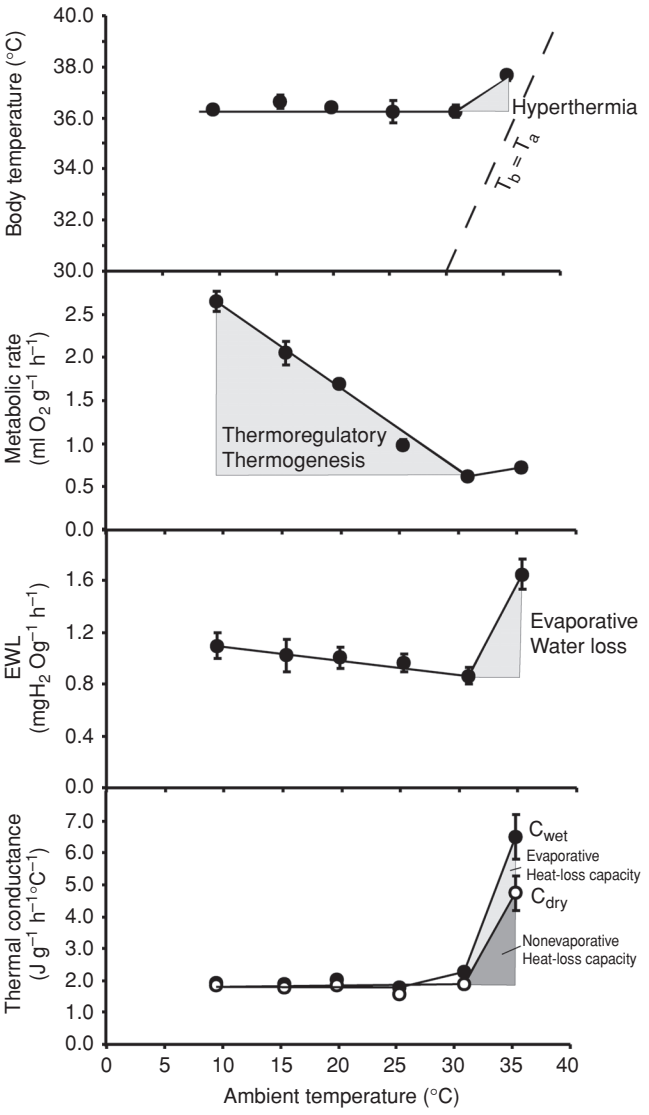


**Figure 7** Pattern of change with $T_a$ for body temperature, metabolic rate, evaporative water loss, and wet (solid symbols) and dry (open symbols) thermal conductance for a typical endotherm, a small marsupial, the dibbler *Parantechinus apicalis* (modified, with permission, from reference 605).

proportion of total body mass; shivering can result in a two to five fold increase in MHP over a period of only minutes (482). Shivering is a generalized thermogenic response of many endothermic groups, including endothermic insects and reptiles, as well as mammals and birds (601).

NST is a second method of increasing MHP. Here, various enzyme systems metabolize fats with the primary purpose of producing heat; very little energy is converted to ATP (165). These cellular mechanisms of heat production are controlled by hormones (catecholamines) and the sympathetic nervous system, and involve organ systems such as adipose tissue, liver, and muscle (363). Brown adipose tissue (BAT or brown fat) is an adaptation for rapid NST in some placental mammals. BAT consists of highly vascularized fat stores with a very high concentration of mitochondria. Heat production in BAT occurs directly in the fat cells, with no requirement for conversion to fatty acids and subsequent transportation and oxidation as occurs with typical adipose metabolism. Therefore, substantial, rapid heat production can occur in BAT, and this heat is then distributed via its extensive vascularization. Two processes are involved in heat production. Firstly, there is a high rate of normal ATP hydrolysis for cellular processes, producing heat. However, additional heat production occurs as a consequence of uncoupling of the normal ATP production that occurs during respiratory oxidation. Normally, ATP is synthesized by the phosphorylation of ADP when protons move down their electrochemical gradient from the cytoplasm into the mitochondria across the inner mitochondrial membrane. However, in BAT, protons "leak" across the inner mitochondrial membrane via specific "uncoupling" proteins called thermogenin or uncoupling protein 1 (UCP1), with the energy liberated as a result of this ion flow producing heat rather than synthesizing ATP. Thermogenesis in BAT is controlled by the sympathetic nervous system, where released noradrenaline binds to receptors on the BAT cells. BAT is only found in some placental mammals, being particularly prominent in those that require large, rapid rates of heat production, such as small species, especially those that arouse from torpor or hibernation, and neonates (363, 482). BAT has not been positively identified in monotremes, marsupials or birds (234, 274).

There is some evidence for NST in some marsupials, such as in the bettongs, often referred to as rat-kangaroos (622) and the Tasmanian devil (284), but not others (401). Shivering is the primary mechanism of heat production in birds but there is some evidence for NST (154). NST appears to occur in skeletal muscle, adipose tissue, and other organs such as the liver, but the cellular mechanisms for NST in tissues other than BAT are not well understood. There is a complex distribution of mitochondrial uncoupling proteins (UCP1, UCP2, and UCP3) in many vertebrates, from fishes to amphibians, birds, and mammals (monotremes, marsupials, and placentals; 261, 274). In fish, UCP1 is present in the liver, kidneys, and brain, but its physiological role is uncertain; in mammals, UCP1 is found in adipose tissue (275) and has a thermogenic role as described previously. It seems that an archetypal BAT-like thermogenic tissue was present early in the evolution of mammals, before the divergence of marsupials and placentals (274). Acclimation to cold stimulates the thermogenic response in some mammals, and often shivering is replaced by NST after acclimation (136, 284, 401, 441, 450).

## Radiative heat gain in endotherms

Endotherms can exploit external heat sources to reduce the energetic costs of thermoregulation at low $T_a$. Exposure to solar radiation can reduce metabolic rate at $T_a$ below thermoneutrality due to absorption of solar energy as heat, and a reduction (or even reversal) of $\Delta T$ (363). Basking dunnarts reduce their energy expenditure at low $T_a$ by up to 74% compared to dunnarts with no heat source (584). Ground squirrels can absorb 14% to 22% of incident solar radiation, reducing MHP by up to 42% (578). Like ectotherms, endotherms may have behavioral and/or anatomical adaptations to enhance solar heat gain and reduce thermoregulatory requirements. The diurnal marsupial numbat (also known as the banded anteater) has traded off thermal insulation for enhanced solar heat gain, with a shallow, sparse pelt that facilitates 60% to 63% of incident solar radiation acting as a heat load on the skin. Its activity is also positively correlated with daily and seasonal variation in ambient light intensity (102, 103). Striped mice trade off foraging and sun basking depending on energy availability, forgoing basking on cold mornings during periods of high food abundance, but delaying foraging in favor of sun basking at the burrow when food is less abundant (458). Color morphs that exist within larger mammals (such as the springbok) also demonstrate that low reflectance (i.e., black) color morphs absorb significantly more solar radiation, leading to lower metabolic costs of feeding (244). Further examples on the impacts of reflectance changes on thermal balance in animals are provided in the section on ectotherms later.

## Heat-conservation mechanisms

Heat loss can be reduced by either decreasing $T_b$ (and therefore $\Delta T$; see later text), or by decreasing thermal conductance. Thermal conductance (C) is the inverse of insulation, and is a measure of the rate of heat exchange between an animal and its surroundings depending on surface area, the temperature differential between the animal and its external environment ($\Delta T$), and the insulative properties of the animal's surface (fur or feathers). Thermal conductance can be calculated for an endotherm as metabolic heat production (MHP) per $\Delta T$, that is, $C = MHP/(T_b\text{-}T_a)$, where $T_a$ is the ambient temperature. This is more correctly termed wet thermal conductance ($C_{wet}$) because it includes heat loss through evaporation (see later). Body size influences C, as smaller animals have a higher surface area to volume ratio than larger animals. Smaller animals will therefore have a higher rate of heat exchange with their environment relative to their size (mass). Birds have a lower thermal conductance ($C = 0.687$, $M^{-0.477}$; 452) than

marsupial ($C = 0.914\ M^{-0.463}$) and placental ($C = 1.02\ M^{-0.505}$) mammals (342). The allometric slopes for these relationships of about −0.50 are not equal to the exponent for mass-specific metabolic rate (about −0.25) because of the relationship between surface area and body mass. The different slopes for metabolism and conductance are related to an allometric effect on the lower critical temperature ($T_{lc}$) of the thermoneutral zone; for birds, $(T_b - T_{lc}) \propto M^{-0.22}$ hence $C \propto M^{-0.47}$ not $M^{-0.25}$ (452).

Bergmann's rule (see Section "Bergmann's rule") proposes that endothermic species are larger at higher latitudes; this may be a consequence of selection for a larger body mass and decreased mass-specific thermal conductance in colder climates (e.g., wood rats, puma, and penguins; 62, 362, 482). Another bioclimatic rule, Allen's rule (see Section "Allen's rule"), which states that species have smaller appendages in cold climates, also reflects conservation of body heat at low environmental temperatures. The reduced surface area to volume ratio of smaller appendages presumably also reduces loss of metabolic heat. For example, foxes and lagomorphs have smaller ears in colder climates than in warmer environments (243, 601). Postural adjustments may assist in conserving $T_b$. A sphere has a smaller surface area to volume ratio than other shapes and so is the most effective body shape to conserve heat. Consequently, many animals adopt a spherical posture when resting at cold temperatures. Appendages often have a relatively large surface area and poor insulation, so animals generally retract or cover their appendages in the cold, for example, birds draw feet and limbs under their feathers and tuck their bills under their wings, and mammals curl up and draw in their limbs. Thermal conductance may be reduced by increasing thermal insulation. Thermal conductance of an insulating layer is a consequence of both the surface area through which heat is flowing (A), thermal conductivity of the insulating material (k), and thickness of the insulating layer (x); $C = kA/x$ (601). Thermal conductivity is a physical property of the insulation; it ranges from 0.024 J $m^{-1}$ $°C^{-1}$ $s^{-1}$ for still air to more than 0.2 J $m^{-1}$ $°C^{-1}$ $s^{-1}$ for some biological materials (see reference 601). Therefore, still air is the best insulating material available to animals, and many endotherms have an insulating layer of fur or feathers that efficiently traps a layer of still air close to the body (Fig. 8). Feathers are more effective at trapping air than fur, so birds have approximately a 15% lower C than mammals (363, 452). Increasing the thickness of the insulating layer reduces C, so in the cold mammals pilo-erect their fur and birds ptilo-erect their feathers to trap a thicker layer of still air and reduce heat loss. There are strong relationships between climate and insulation for birds and mammals. Arctic mammals have better insulation than tropical mammals, due to a thicker and denser pelt, and many species seasonally adjust C to minimize heat loss during cooler periods (76, 229, 456). For very small mammals however, there is a limit to their use of insulation to retard heat loss as they cannot support a thick fur pelt or carry a large amount of body fat (363).

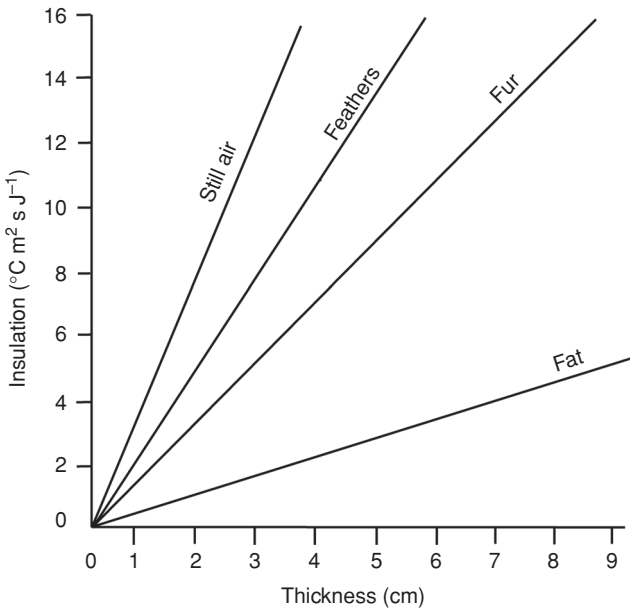


**Figure 8** Effect of insulation thickness on thermal conductance of still air, feathers, fur, and fat. Modified, with permission, from references 456 and 601.

Aquatic endotherms often use a subcutaneous layer of fat (blubber) for insulation despite its relatively high thermal conductivity (0.17 J $m^{-1}$ $°C^{-1}$ $s^{-1}$) compared to still air (0.02 J $m^{-1}$ $°C^{-1}$ $s^{-1}$). Fur and feathers are typically not effective insulators when submerged in water (exceptions being sea otters) as they become compressed (reducing thickness), cannot be pilo/ptilo-erected, and water has a higher specific heat (4.2 J $g^{-1}$ $°C^{-1}$) and thermal conductivity (0.59 J $m^{-1}$ $°C^{-1}$ $s^{-1}$) than air (1.0 J $g^{-1}$ $°C^{-1}$; 363, 601). A low density of capillaries in the insulating blubber aids in retarding heat loss to the water.

External insulation, such as nesting material, may further retard heat loss, with substantial energetic benefits. For example, the grass and twig nest of a numbat (marsupial anteater) has a thermal conductance of 1.2 J $g^{-1}$ $h^{-1}$ $°C^{-1}$, which effectively doubles the numbat's whole body insulation and results in a nightly energy saving of 20kJ compared to resting in a burrow with no nest (103). Similarly, lemming nests reduce their overall thermal conductance (75). For communal species, huddling can reduce the C of the group considerably. Below thermoneutrality, the total metabolic rate of a group of huddling birds or mammals is less than the sum of the metabolic rates of each exposed individually to that $T_a$ (363). This energy savings is a consequence of reduced heat loss across surfaces in contact with another individual; the group of animals essentially has a higher "body mass" hence a lower mass-specific thermal conductance than an individual. For example, a sugar glider in a group of four at 15°C has the same metabolic rate as at a thermoneutral $T_a$ (180), and huddling in a group of four and having nesting material reduced the metabolic rate and thermal conductance of naked mole rates by about 80% (606).

## Heterothermic mechanisms

The energetic consequences of maintaining homeothermy at low $T_a$ can be prohibitive for many birds and mammals, particularly those inhabiting regions or occupying niches where food may be scarce during cold periods. Cold is even more physiologically challenging for small species with a large surface area to volume ratio and limited capacity to store energy reserves in the form of body fat. Heterothermy may provide them with an energetically favorable mechanism for dealing with cold, as reducing $\Delta T$ reduces the thermal differential driving heat loss and consequently reduces their energetic requirement; a lower MHP is required to defend a smaller $\Delta T$, and there are energetic reductions associated with a lower $T_b$ due to the $Q_{10}$ effect.

Peripheral or spatial heterothermy is one approach to heterothermy that is used by essentially homeothermic animals to conserve energy; the animal maintains core $T_b$ at a normothermic level, but peripheral tissues are allowed to cool considerably, resulting in reduced heat loss across their surface. For example glaucous-winged gulls standing on ice may have a core $T_b$ of 37.8°C but a foot temperate of only 0°C; porcupines at $T_a$ of −43°C maintain a core $T_b$ of 36°C but foot temperature may be as low as 3°C; sled dogs at $T_a = -55$°C have a foot temperature of 0°C but a core $T_b$ of 37.2°C (264), and arctic wolves maintain footpad temperatures of −1°C to minimize heat loss while preventing tissue freezing (242). The mechanism for peripheral cooling is reduced blood flow to the periphery, particularly appendages, via vasoconstriction, and a countercurrent heat exchange system between the periphery and the animal's core. This countercurrent exchange system consists of a close association of veins draining blood from, and arteries supplying blood to, an extremity, such as a limb. Heat from warm arterial blood leaving the core is transferred by conduction to cold venous blood returning from the extremity, maintaining a thermal differential between the body and the limb. Such countercurrent heat exchange mechanisms have been identified in a wide range of mammals and birds, but appear more efficient in species inhabiting cold environments (367, 368).

A more extreme approach to heterothermy is hypothermia, a reduction in core $T_b$ as occurs during torpor or hibernation. Adaptive hypothermia is a precisely controlled physiological adaption, which is distinguished from pathological hypothermia by the ability to rewarm spontaneously to normothermia. Most birds and mammals will become pathologically hypothermic once their ability to maintain a constant, normothermic $T_b$ by MHP is exceeded. However, some birds and mammals use adaptive hypothermia to reduce the energetic costs of thermoregulation at low $T_a$. Moderate hypothermia is a small (usually $< 5$°C) drop in body temperature, which is relatively common amongst diurnal birds during their rest phase (360). Torpor and hibernation are more pronounced drops in body temperature (usually $> 5$°C, or $T_b < 30$°C) associated with a decrease in responsiveness and reduction in metabolic rate to below basal levels (604). Torpor occurs for periods less than 24 h, and is usually shallower and results in smaller energy savings than hibernation, which occurs for weeks or even months and results in more substantial declines in $T_b$ and larger energy savings.

Torpor (and hibernation) results from the lowering of the normal thermoregulatory set point to a torpor set point. At $T_a$ above this torpor set point, torpid animals abandon thermoregulation and passively conform to $T_a$. However, when $T_a$ is below the torpor set point, proportional metabolic thermoregulation is initiated and $T_b$ is defended at the torpor set point, just as during normothermia. The thermoregulatory consequences of torpor can be substantial. For many marsupials, daily torpor involves a decrease in body temperature from normothermia ($T_b = 35$°C) to $T_b = 11$ to 28°C with a concomitant reduction in metabolic rate to 10% to 60 % of basal metabolic rate. During hibernation $T_b$ may drop even lower, to 2 to 5°C, and metabolic rate may be only 2% to 6% of basal metabolic rate (195). Body temperature set point is generally maintained above the freezing point of tissues, although arctic ground squirrels allow $T_b$ to drop below 0°C and rely on supercooling to prevent freezing at a $T_b$ of −2.9°C (33).

The greatest reduction in energy expenditure during hypothermia results from the absence or reduction in thermoregulatory heat production due to a lower $T_b$ set point (321, 322), combined with a reduction in metabolic rate due to the $Q_{10}$ effect of low $T_b$. It is possible that some torpid endotherms further depress metabolic rate beyond these simple thermoregulatory and biochemical effects. A $Q_{10}$ for metabolic rate above three for some hibernating species (e.g., some bats) suggests that there may be additional biochemical depression of metabolic rate, although the energetic consequences of any intrinsic depression are likely to be very small relative to the aforementioned effects (194).

Energetic advantages of torpor or hibernation are somewhat offset by the energetic costs of rewarming to normothermia. Spontaneous rewarming from hypothermia may require a 100-fold increase in metabolic rate, although behavioral mechanisms such as solar basking can offset some of these costs (197, 198). Rewarming costs are one reason why hypothermia is more common amongst, and is deeper for, small mammals. It takes 3.9 J to warm 1 g of tissue by 1°C (601), so arousal is more energetically expensive for large compared to smaller species, and cooling and rewarming rates are faster for smaller species due to a smaller thermal inertia (101, 196). The ratio of heat production in the peripheral tissues to available surface area for heat exchange is lower in larger mammals (52), which further complicates the speed with which arousal or rewarming can occur with increase body size (601).

Torpor has evolved independently in both birds and mammals, although it appears to be more common amongst mammalian than avian species. For some groups such as bats and marsupials there are strong phylogenetic patterns to use of torpor, but for others, such as rodents, use of torpor and hibernation seem to be more closely related to environmental demands than a shared phylogenetic history (100).

# High Temperature Adaptations in Endotherms

## Passive mechanisms: Temperature differentials

Endothermic mammals and birds characteristically maintain a relatively constant $T_b$ by physiological mechanisms that are often supplemented by behavioral means. For an endotherm at cold temperatures, the thermal differential for heat loss ($\Delta T = T_b - T_a$) is high, and this high rate of heat loss can be counterbalanced by elevated MHP or decreased thermal conductance to keep $T_b$ constant. In contrast, heat loss is compromised at high ambient temperatures by the reduced thermal gradient; the $\Delta T$ can become inadequate to dissipate MHP even if $T_b > T_a$, and promotes heat gain from the environment if $T_b < T_a$.

There are three basic strategies for coping with high $T_a$. The first is to tolerate an increase in $T_b$ so that the thermal differential is maintained at the minimum required to dissipate MHP passively. The second is to regulate a relatively constant $T_b$ by increasing the rate of heat loss; evaporation of water is the only physiological mechanism available for an animal to dissipate heat against a thermal differential. The third is to modify the rate of heat exchange between the animal and its environment (thermal conductance) and rely on thermal inertia to keep $T_b$ from increasing to a critical level.

## Hyperthermia and thermal tolerance

Thermal tolerance is a fairly common strategy for mammals and birds, particularly at moderately elevated $T_a$s, or in concert with other strategies (see reference 550 for a review of hyperthermia in birds). Allowing $T_b$ to increase passively (hyperthermia) can maintain a sufficient thermal differential to dissipate MHP (Fig. 9). For example, an endotherm could "thermoconform" to keep a constant $T_b - T_a$ of 2.5°C to dissipate MHP at elevated $T_a$. In contrast, thermoregulating at a constant $T_b$ will reverse $\Delta T$ and add an environmental heat gain to the MHP. Many endotherms are close to "thermoconforming" at high $T_a$ (e.g., the European starling has a $T_b$ of 45.8°C at a $T_a$ of 45°C; Fig. 9) whereas others are good thermoregulators (e.g., the Monk parakeet has a $T_b$ of 41.1°C at a $T_a$ of 45°C). Hyperthermia also has the short-term (non-steady state) benefit of storing heat in the body tissues as they warm (see later). The specific heat is about 3.9 $J \cdot g^{-1} \cdot {}^{\circ}C^{-1}$ for biological tissues, so a considerable amount of heat is stored when tissues are warmed and $T_b$ increases; the stored heat is lost when the $T_b$ subsequently declines. Hyperthermia can store about 2% to 6% of basal heat production of mammals and birds in the short-term (dependent on body mass and time; see reference 601). Another benefit of hyperthermia is that it decreases the thermal differential for heat gain from the environment. A concomitant advantage of hyperthermia is the conservation of body water by reducing the evaporative water loss required for thermoregulation. For example, hypothermia at $T_a$ above thermoneutrality enables the kaluta, a small arid-habitat marsupial, to save about 11 mg $H_2O \cdot g^{-1} \cdot h^{-1}$ by reducing EWL to only 57% of what would be required to maintain normothermia (603).

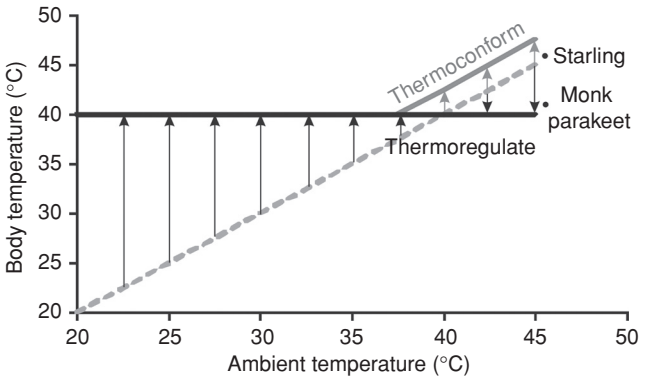


**Figure 9** Schematic representation of the difference between an endotherm thermoconforming to keep the thermal differential ($\Delta T$) constant at elevated $T_a$ compared to thermoregulating at a constant $T_a$. Examples of birds (see reference 550) thermoconforming (starling) and thermoregulating (Monk parakeet) are also shown.

The dromedary camel is a classic example of an endotherm that uses hyperthermia to store heat and reduce the thermal differential for heat gain, especially when it is dehydrated and body water must be conserved (453). For a dehydrated camel, hyperthermia of 6°C through the day stores about as much heat as is gained from the environment and evaporation dissipates about as much heat as is produced by metabolism. In marked contrast, a hydrated camel only allows $T_b$ to increase by 2.1°C, gaining about three times as much heat from the environment and having to dissipate most of this heat by evaporation. A possible disadvantage of hyperthermia is that $T_b$ rises, and this might result in a reduction in physiological performance. There will also be an increase in metabolic rate by a $Q_{10}$ effect, hence an increase in MHP. An obvious limitation to hyperthermia is that eventually a critical $T_b$ will be reached and the animal will die. However, a large mammal, like a camel (about 260 kg), has sufficient thermal inertia that it can tolerate progressive hyperthermia for the entire day, before radiating the stored heat to the night sky. Smaller mammals do not have this temporal luxury. For example, the antelope ground squirrel (about 80 g) has a much lower thermal inertia and a higher mass-specific MHP than a camel, and consequently has a much shorter period over which it can store heat (about 10-20 min), so it needs to escape by seeking shade or returning to its burrow (80; Fig. 10A).

## Evaporative heat loss

Thermoregulating to maintain a constant $T_b$ is the opposite strategy to "thermoconforming" and becoming hyperthermic; when $T_a > T_b$, $T_b$ is kept constant by enhanced evaporative heat loss. The latent heat of vaporization is about 2400 J/g of water at biological temperatures, so 1 gram of water dissipates the metabolic heat arising from the consumption of about 120 mL $O_2$ (601). However, thermoregulation reverses the $\Delta T$ if $T_a > T_b$ and promotes passive heat gain from the environment, which must be dissipated in addition to the metabolic


Figure 10 (A) Antelope ground squirrel (*Ammospermophilus leucurus*) dissipating heat in the shade (photo C.E. Cooper). (B) Red kangaroo (*Macropus rufa*) licking its forearms for evaporative heat dissipation (photo A. Lothian). (C) Facial view of eland showing engorged nasal veins returning blood to the angularis oculi and facial veins (photo A. Fuller).

heat load, thereby exacerbating the required evaporative heat loss. Nevertheless, many endotherms use evaporative cooling to thermoregulate when heat challenged. This enhanced heat dissipation is evident from the increase in $C_{wet}$ of endotherms when heat challenged. For most endotherms, the rate of evaporative water loss increases at high $T_a$, evaporative heat loss increases, and $C_{wet}$ increases (e.g., Fig. 7).

The mechanism for enhancing evaporative cooling varies quite markedly amongst different endotherms. Many endotherms salivate and drool or spread saliva over parts of their body when heat challenged. For example, many marsupials salivate profusely when heat stressed (e.g., reference 436), as do some placentals (particularly rodents), especially at extremely high $T_a$. However, salivation does not occur in other placental mammals such as sheep (221, 454, 597). A few birds may salivate (35, 166, 248), but this is unlikely to have thermoregulatory significance. Salivation is sometimes interpreted as a "primitive" and unsophisticated thermoregulatory strategy, but licking can be a quite sophisticated thermal adaptation, with anatomical and physiological specializations, and behavioral coordination. Large kangaroos, for example, have a dense superficial network of veins beneath the skin of their forelimbs, which they lick when heat stressed (Fig. 10B; 388, 389). Panting is also a common method of enhancing evaporative heat loss. Respiratory ventilation is increased by elevated respiratory frequency, often to a very high resonant frequency with decreased tidal volume. There is a concomitant decrease in oxygen extraction reflecting the enhanced evaporative rather than gas exchange role of ventilation during heat stress. A decreased tidal volume can reduce alveolar ventilation and avoid or minimize hypocapnia (e.g., reference 352). Birds can pant or may gular flutter, where the moist gular (throat) region is moved by the hyoid apparatus in synchrony with, or independent of, panting. The efficacy of panting is increased by reducing the efficiency of nasal countercurrent heat/water exchange, for example, by mouth breathing or an increased blood flow to the nasal mucosa. An alternative mechanism for enhancing evaporation is cutaneous sweating (many but not all mammals; 182, 276, 597) or general enhanced cutaneous evaporation (some birds such as columbiforms; 47, 353, 354). Two final unusual mechanisms for enhancing evaporative water loss are evaporation from the cloaca, for example, the Inca dove and Eurasian quail (Hoffman et al. 2007), and urohydrosis, urination on the body to promote evaporation, for example, wood stork, turkey and black vultures, and gannets (14, 104, 285).

## Altering thermal conductance

Another physiological strategy for coping with heat stress is to modify the rate of thermal exchange with the environment to either facilitate nonevaporative heat loss or decrease the rate of heat gain (e.g., from solar radiation). Dry thermal conductance is often high for tropical, desert, fossorial, or large endotherms to facilitate nonevaporative heat dissipation (363). If $T_b$ remains higher than $T_a$, then more heat

can be dissipated if the resistance to passive heat exchange is lowered, that is, if the dry thermal conductance is increased. Many endotherms increase dry thermal conductance when heat challenged [e.g., the dibbler, a small marsupial (Fig. 7)]. $C_{dry}$ can be increased by postural changes, pilo-depression of the fur or ptilo-depression of feathers, or increased blood flow to the skin. Particular body surfaces can be specialized as "thermal windows" that promote heat loss by conduction, convection, or radiation to the environment. Examples of specialized thermal windows include the large ears of jack rabbits and elephants (245, 414), the relatively naked under surfaces and legs of camels, emus, and ostrich (345, 415) and the bird bill (Fig. 11; 460, 533). Recent paleophysiological analysis has even noted the importance of thermal windows to extinct dinosaurs, such seen in the plates of the *Stegosaurus*. (174). An insulating pelage (fur or feathers) can absorb radiant energy near the surface, and allow some of the heat to be lost to the environment by conduction, convection, and radiation. This insulating layer of pelage is called a heat shield, and can reduce the solar heat gain for a variety of birds and mammals, for example, ostrich, emu, camel, and sheep, (135, 335, 346, 454, 607). In this scenario, an increase in the pelt thickness (decreased thermal conductance) and black coloration of a thermal shield can be beneficial. For example, the pelage (feathers) of the common pigeon has a lower conductance when ptilo-erected, and a lower solar heat gain (577). However, the efficacy of the heat shield and the role of color also depend on environmental conditions, such as wind speed. Solar heat gain by pigeon pelage is higher for black feathers when ptilo-erected than for white at low wind speeds ($< 5$-$6\ m{\cdot}s^{-1}$) but is lower for black than white at higher wind speeds; ptilo-depressed black feathers had a higher solar heat gain even at $8\ m{\cdot}s^{-1}$ (577).

There are also a variety of behavioral strategies for avoiding an environmental heat load, including resting to minimize endogenous heat production, shade seeking, retreat to burrows or switching to nocturnal activity to avoid a solar heat load, and wading into water to conductively/convectively dissipate heat. Many small diurnally active mammals, such as ground squirrels, seek shade when the heat load becomes excessive (e.g., Fig. 10A). The black wildebeest inhabits the treeless plains of hot, arid regions of Southern Africa, so shade seeking is not an option to reduce solar heat load; its solution is to orient its long axis of the body towards incoming solar radiation, reducing radiant heat load by about 30% (350). The dipper, a semiaquatic bird, relies on its legs to dissipate heat to cold water when heat stressed (382).

## Selective brain cooling

The central nervous system is often regarded as a critical organ that is susceptible to damage by heat. In the 1960s, it was discovered that many mammals possess a system that enables selective brain cooling (SBC) and that ability is seen as an important adaptation to hot, arid regions. Recently, it has been shown that this system does not operate to "protect" the brain, but may provide advantages in reducing water use in hot environments.

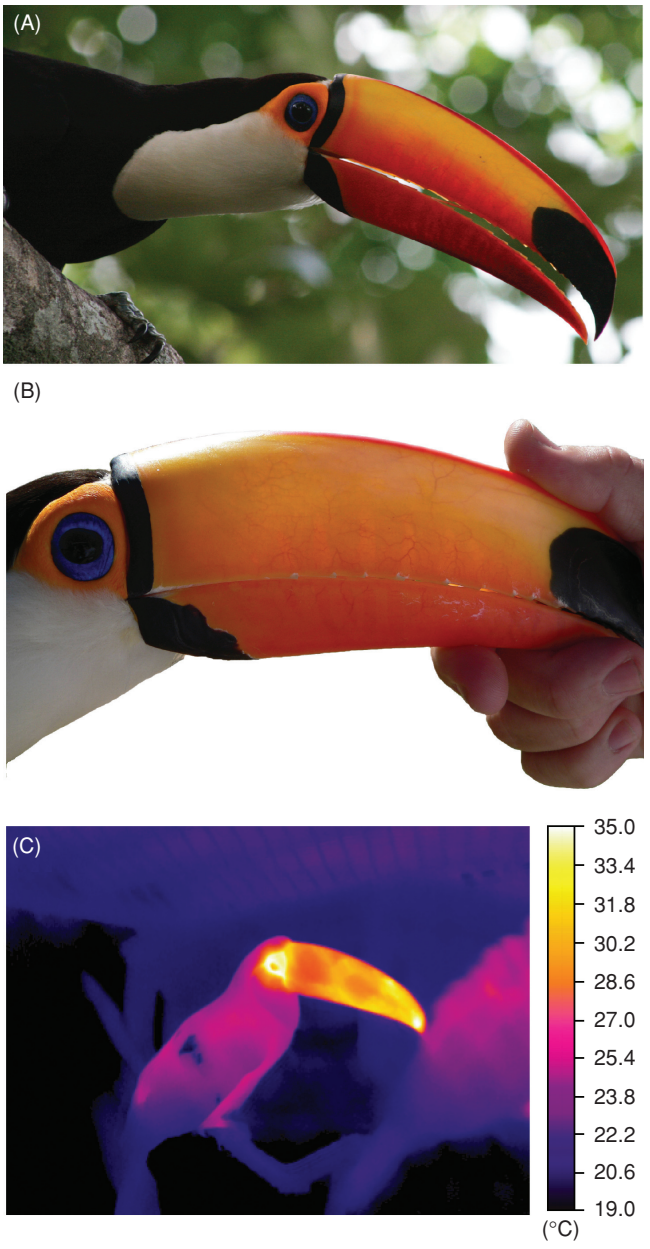


**Figure 11** Toco toucan (A) (live image) utilizes the underlying vasculature within its bill (B) (live image) as a thermal window to lose heat (C) (thermal image). Photos courtesy of G.J. Tattersall.

The major factor influencing the temperature of the brain is the temperature of the blood reaching it (233). Very little of the brain's metabolic heat is lost across the cranium, with most of it being lost in the venous blood, meaning that the brain always is hotter than the arterial blood supplying it. The temperature at sites within the brain depends on the interaction of local metabolism (heat production) and blood flow. One way for animals to regulate their brain temperature is thus to regulate the temperature of the blood reaching the brain. A sweating human is "brain cooling" because the evaporation

of sweat removes heat from blood perfusing the skin, cooling the body core, and resulting in a cooling of blood going to the brain, and thus cooling of the brain itself. If that human were not sweating then blood and brain temperature would be rising in parallel as hyperthermia developed.

Although selective brain cooling has been demonstrated in circumstances that also lead to whole body cooling (namely hypoxia-induced anapyrexia; 541), some mammals, notably the artiodactyls (even-toed ungulates) and felids, are capable of "selective brain cooling," defined as having a brain temperature lower than the simultaneously measured arterial blood temperature (10). These species have an intricate vascular anatomy, the rete mirabile (wonderful net) or "carotid rete" (26) where the main blood supply to the brain, the carotid artery (internal carotid in some species, external carotid in others) divides into a plexus of fine arteries of diameter 250 to 500 μm. The rete lies within a venous sinus, the intracranial cavernous sinus in artiodactyls and the extracranial pterygoid sinus in felids. Venous blood in the sinus originates from many sites in the head, including the nasal mucosa where the blood has been cooled by evaporation (Fig. 10C). The high surface area of contact between arterial blood in the rete and the venous blood within the sinus, and thin walls of the rete vessels, facilitates heat transfer and results in cooling in the rete of the blood destined for the brain. The relationship between postrete blood, and brain temperature in mammals with a rete is no different to that in mammals without a rete; the difference is that the blood that perfuses the brain has been cooled before it reaches the brain. In nonrete species, notably the perissodactyls (odd-toed ungulates), rodents and primates, the carotid artery does not ramify, though it does traverse a venous sinus.

When it was first discovered, selective brain cooling was interpreted as an important adaptation to heat stress. During hyperthermia, especially that associated with exercise, selective brain cooling was proposed to protect the brain from thermal damage. In laboratory studies of several species, that hypothesis was supported because there is a threshold core temperature above which selective brain cooling is activated, indicating that above a certain threshold the brain is thermally protected (as indicated by the "mean" points in Fig. 12 that are lower than blood temperature above 39°C). The mechanism for selective brain cooling involves controlling the supply of cool venous blood to the sinus (see reference 370) and the stimulus is brain (most likely hypothalamic) temperature (280,306,307). At low core temperatures, the veins supplying venous blood to the rete are constricted, and when core temperature increases those veins dilate and supply cool blood to the sinus, resulting in greater heat loss from the arteries supplying the brain, and thus selective brain cooling. While the bilateral ablation of the angularis oculi veins in sheep resulted in a reduction in the magnitude of selective brain cooling, they retained the ability to control the process (187), indicating that the flow of cool blood via other routes to the sinus is under physiological control, and can be controlled in the same manner as has been shown for the oculi veins.

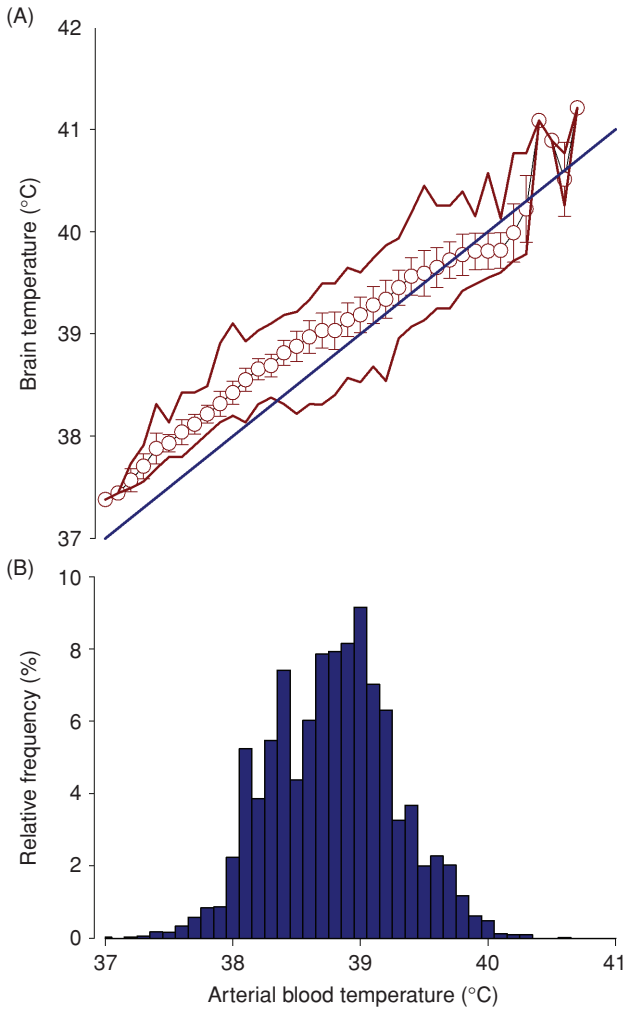


**Figure 12** Body (arterial blood and brain) temperatures of four free-ranging Southern oryx measured every 5 minutes for 3 months. The top panel (A) shows the mean ± SD, with the lines denoting maximum and minimum, brain temperature at each 0.1°C category of arterial blood temperature measured in the carotid artery. The lower panel (B) shows the frequency of occurrence of each arterial blood temperature category. The diagonal line in the top panel is the line of identity; points above the line indicate the brain was hotter than the blood, points below the line denote selective brain cooling.

In the early 1990s, the technology became available to measure blood and brain temperatures very accurately in wild, free-living mammals. Jessen et al. (278) instrumented and released several black wildebeest in South Africa to measure temperature in the carotid artery and brain every 2 min for several months. Three things were immediately obvious from their data. Firstly, in contrast to the laboratory where selective brain cooling seemed to be activated at a given core temperature and increased the higher core temperature became, in the field there was a wide range of core temperatures over which selective brain cooling was sometimes present, including at normothermic temperatures. A similar pattern is evident for the oryx (Fig. 12), where brain temperature varied widely in the normothermic to hyperthermic range, and that the brain

could be cooler or warmer than the blood at 38°C, and even at 40.5°C the brain could be hotter than the blood. Secondly, the frequency histogram of core temperatures showed that the wildebeest very rarely became dangerously hot and when they did it was associated with strenuous activity. Lastly, when the wildebeest were strenuously active, and core temperatures became dangerously high, they did not use selective brain cooling, that is, brain temperature was equal to or exceeded arterial blood temperature.

The same patterns of selective brain cooling have subsequently been recorded for other species in the field, including oryx (Fig. 12), springbok, and eland. On the basis of these results a role for selective brain cooling as a protective mechanism looked tenuous, and so an alternative explanation for its evolution was proposed. When selective brain cooling is activated the temperature of the hypothalamus is reduced. The hypothalamus houses the neural control centers that stimulate heat defense, including evaporative water loss. Jessen (277) proposed that the adaptive significance of selective brain cooling is to modulate the use of water for thermoregulation. Under that scenario, if the conditions are appropriate, an animal will activate selective brain cooling when its core temperature increases and so inhibit the evaporative response, shifting the mechanisms for heat loss to nonevaporative ones (conduction, convection, and radiation) via the skin. In conditions not appropriate for dry heat exchange, such as during strenuous exercise to escape a predator, selective brain cooling is not activated, the hypothalamus is heated, and evaporative heat loss is stimulated fully. Kuhnen (305) showed that goats that used selective brain cooling during heat exposure had a lower water use than goats that did not use selective brain cooling. It seems that the role of selective brain cooling as an adaptation to heat stress is subtler than first thought. It has the potential to reduce water loss, especially when animals are faced with simultaneous heat and water stress. Indeed, it is argued that the rete and the ability to use selective brain cooling underlies the increased diversity of artiodactyls, in contrast to the contraction of perissodactyl diversity since the Eocene (372). If selective brain cooling does serve to modulate water use, then a logical prediction would be that an osmotically stressed animal could reduce water use by augmenting selective brain cooling. Indeed, sheep deprived of drinking water for 5 days and exposed daily to heat have an increase in selective brain cooling and thereby presumably conserve more water (189).

Selective brain cooling in mammals that do not possess a carotid rete, including humans, is contentious (116). There are several confounding factors that can result in erroneous conclusions of selective brain cooling. The use of "core temperature" measures other than arterial blood can be misleading, with rectal temperature being especially unreliable (348), and the use of tympanic membrane temperature as a surrogate for brain temperature is not widely accepted as a reliable approach (398). Further, the comparison of blood and brain temperatures when these temperatures are changing rapidly is an issue, because the biophysics of brain heat exchange results in a thermal lag in the brain that can be interpreted as selective brain cooling (347). In nonrete species such as baboons (349), horses (371), and kangaroos (347) in which arterial blood and brain temperatures have been measured simultaneously over periods of weeks to months to exclude any effect of thermal lag, brain temperature has always exceeded arterial blood temperature even when animals had brain temperatures exceeding 40°C. To our knowledge, there is no unambiguous evidence for selective brain cooling in species that do not possess a carotid rete, but this is contentious and subject to recent debate (see reference 188).

## Ectotherms at Subzero Temperatures

Below freezing, water crystallizes into ice. Ice is less dense than water, and water molecules are unavailable for participation in biochemical processes, so the water-to-ice transition is an extremely important threshold for ectothermic animals. Subfreezing temperatures are encountered in terrestrial polar and alpine habitats year round, and seasonally in temperate and even subtropical environments. In addition, because solutes in seawater depress its freezing point to approximately −1.86°C, temperatures below their freezing point are encountered by marine organisms in polar and temperate oceans, while temperate intertidal organisms may be exposed to freezing temperatures at low tide during the winter (140). The formation of internal ice has the potential to cause severe damage to tissues, cells and proteins, both through mechanical damage, as well as the effects of osmotic concentration and anoxia.

Although the melting point of pure water is, by definition, 0°C, the freezing point (the temperature at which ice forms in solution) can be much lower, depending upon temperature, the time spent at that temperature, the presence of solutes, and the volume of water. The presence of solutes colligatively depresses the melting point of a solution at a rate of $1.86°C \cdot mol^{-1}$ of solute (bearing in mind that ionic compounds like NaCl will dissociate in solution to provide two solutes, $Na^+$ and $Cl^-$), and also have an effect on the freezing point. Freezing of a solution, while theoretically at the melting point, is generally lower (sometimes substantially so), and is dependent upon the spontaneous formation of an ice-like template by water molecules about which a crystalline structure can form (183). Heterogeneous particles that cause this arrangement (ice nucleating agents) can also increase the freezing point, and be of biotic (e.g., the ice nucleator from the bacterium *Pseudomonas syringae* used as a reporter gene; reference 3) or abiotic (e.g., silver iodide crystals; reference 598) origin. Ice crystals themselves are extremely effective ice nucleators. A solution that remains liquid at temperatures below its melting point is said to be supercooled (Fig. 13). This is a metastable state, and the rate of freezing of a supercooled solution is dependent on the degree of supercooling, which is theoretically possible in an aqueous solution to about −54 °C (183). When ice forms, only water is incorporated into the ice lattice, resulting in concentration of other solutes in the remaining solution. Once frozen, ice crystals exchange

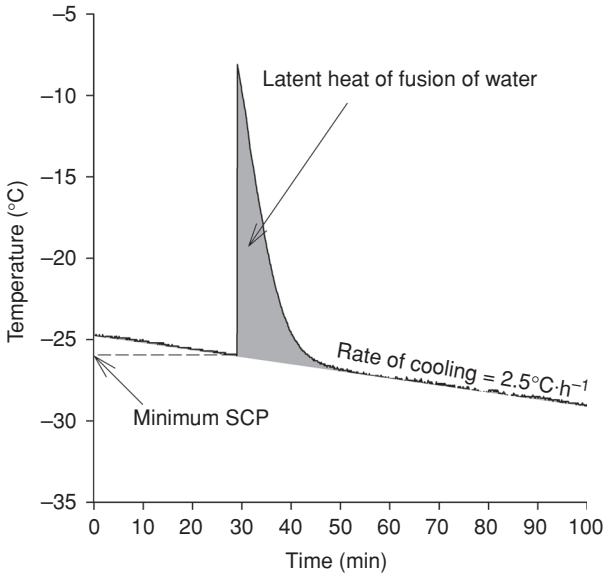


**Figure 13** Body temperature profile of an insect undergoing freezing, demonstrating the minimum supercooling point (−26°C) where ice nucleation occurs, the heat released (shaded area) from the latent fusion of water, and the background cooling rate (2.5°C·h$^{-1}$). (Unpublished Data, G.J. Tattersall.)

water molecules with their environment, and large ice crystals (which are in a lower energy state) tend to accumulate water molecules at the expense of smaller crystals in a process called recrystallization. The rate of recrystallization is higher at higher subzero temperatures (297).

While some ectotherms can withstand the formation of internal ice (freeze tolerance), many cannot and rely on maintaining their body fluids in a supercooled state (freeze avoidance), or removing freezable water (cryoprotective dehydration). However, the majority of ectotherms have little or no tolerance of subzero temperatures, and mortality in these organisms (chilling injury) is unrelated to ice formation.

## Chilling injury

### *Insects*

Chilling injury can occur both above and below zero, and at subzero temperatures has been divided into acute and chronic chilling injury. Chronic chilling injury can manifest over very long periods (e.g., reference 559), and it is unclear whether the mechanisms underlying chronic and acute chilling injury are related (498). The ability to tolerate chilling may be changed through acclimation and acclimatization (313). In addition, rapid cold hardening (RCH) is a very quick process where acute chilling tolerance may be increased within a few minutes of a prior exposure (141). The causes of chilling injury are poorly known in insects, but are thought to be associated with membrane phase transitions or equilibration of ions leading to membrane depolarization (298, 302). Inducible heat-shock proteins are also associated with cold exposure in insects (e.g., reference 301), which implies that protein misfolding or damage may also be associated with chilling injury. Low temperatures induce apoptosis in *Drosophila melanogaster* adults, and this apoptosis is blocked by rapid cold-hardening (624). Recent work on the fall field cricket, *Gryllus bimaculatus*, suggests that when insects are cooled, transmembrane ion pumps fail, allowing equilibration of ion concentrations between the hemolymph and the gut, and the efflux of hemolymph water to the gut. This in turn alters membrane potentials in calls, possibly leading to irreversible chilling injury (340).

### *Vertebrates*

Vertebrate ectotherms also enter chill coma and may be killed by prolonged exposure to low temperatures that do not result in ice formation. Resistance to chilling has a genetic basis in fish (94). Although most marine animals are not exposed to subzero temperatures, polar species certainly are, and Pörtner (418) has proposed that lower lethal limits in these species may be determined by a mismatch between oxygen supply and demand, driven in part by the limitations of oxygen delivery systems. This theory appears to be well supported in marine species (419), but has not been examined closely in terrestrial ectothermic vertebrates.

## Freeze tolerance

Freeze tolerance is the ability to withstand the formation of internal ice. Generally, ice is thought to be restricted to extracellular compartments (but see exceptions, later).

### *Freeze tolerance in marine invertebrates*

Although polar fishes may be exposed to temperatures 1 to 2°C below 0°C, only marine intertidal organisms are exposed to more extreme subzero temperatures. In the Antarctic and Arctic, ice scour makes such habitats difficult to inhabit, so most cold-hardy intertidal species are found in temperate zones. Because of the presence of abundant water (and therefore ice), cold tolerance strategies veer toward freeze tolerance, and freeze tolerance has been described in intertidal molluscs and barnacles.

Freeze tolerance has been reported in both pulmonate and littorinid intertidal gastropods (334, 498). Few cryoprotectants have been investigated, but hemolymph ice nucleating agents have been reported from the gastropods *Littorina littorea* (19) and *Melampus bidentatus* (343), while *Afrolittorina knysnaensis* is only freeze tolerant if freezing is nucleated externally (496). Holland et al. (251) have shown that cold mortality in *M. bidentatus* is due to rupture of cells. English and Storey (169) showed that metallothionin genes are upregulated in *L. littorea* after freezing, and suggest that this may be a response to oxidative stress during freeze thaw.

A number of bivalve molluscs are freeze tolerant. Kanwisher (287) reports that coastal *Mytilus edulis* may be encased in ice above water in coastal Labrador for up to 8 months per year and that frozen mussels were chipped from

the rocks by local indigenous peoples as a winter food source. Seasonal tolerance to internal ice formation in *Geukensia demissa* (formerly *Modiolus demissa*) appears to be related to an increase in hemolymph calcium (383), as well as the accumulation of anaerobic end products (e.g., proline and alanine), which may also function as cryoprotectants (384). Russell and Storey (443) found reversible depression of carbohydrate metabolism accompanied freezing in *G. demissa*.

Freeze tolerance has been investigated in the acorn barnacle *Semibalanus balanoides* (formerly *Balanus balanoides*), which ranges from temperate shores to the high arctic in the North Atlantic and Pacific (334). *S. balanoides* is freeze tolerant in the winter, surviving the conversion of more than 80% of its body water into ice at temperatures of −18°C (126). This species has strong seasonal cycles in cold tolerance, with freeze tolerance in the winter accompanied by a slight accumulation of glycerol (in the millimolar range; reference 99) and a decrease in saturation of membrane fatty acids (552).

### Freeze tolerance in terrestrial molluscs

Several terrestrial gastropods, including both slugs (529) and snails (11) have shown at least limited freeze tolerance. Temperature and location of ice nucleation appears to be critical to freezing survival, and can be determined by both body size (12) and the presence of bacterial ice nucleators (13).

### Freeze tolerance in nematodes

Both freeliving and plant- and animal-parasitic nematodes may be exposed to subfreezing temperatures. Because of their exposure to ice, and the likelihood of nucleation through the oral, genital and anal openings, many nematodes are freeze tolerant, including endoparasites (e.g., reference 560), free-living stages of parasitic nematodes (590), and free-living nematodes (e.g., reference 591).

Freeze tolerance has been best studied in the free-living Antarctic nematode *Panagrolaimus davidi*, which is one of a few species shown to undergo intracellular ice formation (593). Ice in the intracellular spaces appears to be confined to the cytosol, with something approximating osmotic dehydration preventing ice formation in mitochondria and other organelles (591). Trehalose appears to be important in freeze tolerance of nematodes, as do appropriate cooling and nucleation (594, 596), and, when not directly nucleated, *P. davidi* appears to utilize cryoprotective dehydration (595).

### Freeze tolerance in tardigrades

Although anhydrobiotic tardigrades are famous for tolerating extreme conditions (615), hydrated tardigrades also survive low temperatures (reviewed in reference 507), and calorimetry indicates tolerance of the presence of internal ice (241). This is likely supported by the ability of tardigrades to accumulate trehalose and other carbohydrates (427). Recent evidence also suggests that tardigrade embryos can tolerate internal ice formation under natural conditions (240).

### Freeze tolerance in insects

Freeze tolerance was first observed in caterpillars by Réaumur (431) and species from at least six orders are now known to be able to withstand internal ice formation (140). As much as 82% of body water is converted into ice in freeze-tolerant insects, and some species, once frozen at a higher subzero temperature, can withstand exposure to liquid nitrogen temperatures (140). The accepted model of freeze tolerance in insects sees ice restricted to extracellular spaces, with freeze concentration of the hemolymph resulting in osmotic dehydration of cells, which remain at a concentration that is unfreezable (627). However, survivable intracellular ice formation has been reported in some species, and the importance of extracellular ice formation to survival is poorly understood (497). Many freeze-tolerant insects are able to manipulate ice formation, for example, by the production of ice nucleating agents or the retention of gut contents, although some species only survive freezing if inoculated by external ice, and it is thought that these species are restricted to moist overwintering sites (140). Control of the site and temperature of ice nucleation appears to be one of the few macroscopic differences in ice formation processes between insects that do and do not survive freezing (493).

Freshwater habitats are buffered from air temperatures, but in temperate, polar and alpine environments may freeze—sometimes completely. Aquatic invertebrates are, for the most part, susceptible to inoculative freezing, which suggests that there should be an abundance of freeze-tolerant freshwater species from streams, ponds, even unusual habitats like the fluid of pitcher plants, which contain insects year round. A preliminary investigation of freshwater invertebrates (including insects, as well as a crustacean and a clam) from Ohio suggested that freeze tolerance was not present (185). Recent work by Walters et al. (580) has demonstrated freeze tolerance in an Arctic stonefly.

### Freeze tolerance in terrestrial oligochaetes

Many terrestrial earthworms and enchytraieds are exposed to frost in the soil, and are susceptible to inoculative freezing. At least two species of freeze-tolerant earthworms and several species of freeze-tolerant enchytraeids have been described from temperate and Arctic soils (253,410). Both enchytraeids and earthworms appear to use glucose as a cryoprotectant. Freeze tolerance has been best studied in *Dendrobaena octaedra* (Lumbricidae), and the mechanisms appear to be more akin with vertebrates than with insects. Upon the initiation of freezing from inoculation in the soil, glycogen is mobilized to glucose, providing a cryoprotectant, and osmotically dehydrating cells (253). This glucose is also utilized as a metabolic fuel during the winter (71). Freeze tolerance appears more pronounced in smaller individuals of *D. octaedra*, suggesting

that larger individuals preferentially form cocoons and utilize cryoprotective dehydration for overwintering (255).

### Freeze tolerance in terrestrial vertebrates

Freeze tolerance has been reported in a number of species of terrestrial reptiles and amphibians, including salamanders, hatchling turtles, snakes, lizards, and frogs (112). Hatchling painted turtles (*Chrysemys picta*) can certainly survive internal ice formation (109), but there has been considerable debate as to the ecological significance of this (see 108, 406). Vertebrate freeze tolerance has been best studied in frogs, and follows a pattern similar to oligochaetes. In freeze-tolerant frogs like the wood frog, *Rana sylvatica*, freezing is initiated by ice nucleation through the skin. Upon the initiation of freezing, heart rate increases, and glycogenolysis is initiated in the liver, liberating glucose, which is circulated around the body as a cryoprotectant (113). The organs are osmotically dehydrated by the large quantities of glucose and the bulk of the ice is confined to the coelom (316). Some glucose is metabolized when frozen and during thawing, but much of it is reconstituted into glycogen in the liver, sometimes after being passed into the urine and reabsorbed form the bladder (107). Urea also appears to be utilized as a cryoprotectant in some frogs (111).

## Freeze avoidance

Ectotherms may also survive subzero body temperatures by preventing the formation of ice in the body. This strategy generally relies on supercooling of the body fluids, which in turn relies both on depression of the freezing point and prevention or avoidance of ice nucleation.

### Freeze avoidance by voiding water

A conceptually simple (but physiologically complex) way to avoid internal ice formation is simply to remove body water. Partial removal of body water may serve to increase the concentration of body fluids to the point where the melting point is equal to or lower than the temperature. This strategy, termed cryoprotective dehydration (254) is utilized particularly by soil-dwelling arthropods with permeable cuticles. This strategy is utilized by nematodes (595), earthworm cocoons (257), enchytraeids (508), some Collembola (256, 609, 612) as well as larvae of the Antarctic midge *Belgica antarctica* (167). Some small invertebrates are able to withstand the loss of all of their body water (anhydrobiosis; 128). In the absence of water, these organisms are unfreezable, and, perhaps consequently, tolerate very low temperatures; for example, anhydrobiotic larvae of the midge *P. vanderplankii* can survive immersion in liquid helium (−262°C; 246).

### Freeze avoidance by vitrification

Large concentrations of carbohydrate cryoprotectants can result in aqueous solutions forming a noncrystalline glass-like state when cooled appropriately, and this principle has been used for decades for cryopreservation of cells and tissues (190, 358). Sformo et al. (476) report that the Alaskan beetle *Cucujus clavipes puniceus* is freeze avoiding—some individuals were killed by freezing at temperatures as low as −58°C, while many remained unfrozen (and alive) at temperatures as low as −100°C. Calorimetry was able to demonstrate that the body fluids had undergone a glass transition, the first example of this happening in nature.

### Freeze avoidance in arthropods

Many insects, mites, spiders, and other arthropods, and all insect eggs that have been studied, utilize freeze avoidance as a strategy for surviving subzero temperatures (505). These species generally prepare for winter by emptying the gut, accumulating colligative, and noncolligative antifreezes, and often by moving to hibernacula that allow them to avoid contact with ice; although species in moist hibernacula, like the Emerald Ash Borer *Agrilus planipennis* [Coleoptera: Buprestidae] have cuticles that are resistant to inoculation (127). Freeze avoiding species generally survive low temperatures providing internal ice does not form, so the supercooling point (SCP) is the point of mortality, and measurement of a population distribution of SCPs provides an indication of the distribution of lower lethal temperatures (499). The SCP generally declines in winter, and bimodal distributions of SCPs are often observed; these are thought to be associated with the presence of material in the gut that causes ice nucleation. This approach has been used to particular effect in Collembola, where a number of studies (see reference 73 for a review of earlier studies) have examined seasonal and shorter term changes in distributions of SCPs. In particular, diurnal variation in SCPs of several Antarctic species has been demonstrated (495, 611), although the mechanism remains to be established. There is some evidence that shifts in moulting cycles serve to alter nucleation temperatures in sub-Antarctic Collembola (58, 613).

Most marine invertebrates are isotonic with the surrounding seawater (58), which means they are unlikely to freeze, even in polar environments. An important exception is the fauna of brine channels in polar sea ice, where temperatures may regularly drop well below freezing, and there is inevitable contact with ice nucleators. Recent work by Kiko (291) suggests that *Stephos longipes*, a calanoid copepod that lives in the upper layers of Antarctic sea ice, is freeze avoiding, and that it has a microbial-derived antifreeze protein to facilitate this strategy. Another copepod, *Paralabidocera antarctica* lacks an antifreeze, and is restricted to the thermally stable bottom layers of the sea ice (291).

### Freeze avoidance in molluscs

The majority of cold-hardy terrestrial gastropod molluscs are freeze avoiding, often facilitated by a reduction in water content (11), and at least one intertidal gastropod is freeze avoiding, in spite of an overall trend for freeze tolerance

in this group (496). Although the overwhelming likelihood of inoculative freezing by ice makes freeze tolerance the most common cold tolerance strategy in intertidal molluscs, Antarctic limpets appear to avoid freezing by isolating themselves from ice with a thick layer of mucus (225, 230), and the freeze-tolerant South African littorinid *A. knysnaensis* is freeze avoiding in the absence of external inoculation (496).

### *Freeze avoidance in polar teleost fishes*

The blood of teleost fishes is generally hypo-osmotic to seawater, and as such has a slightly higher melting point (c. −0.7 °C) than seawater. In the polar oceans, seawater temperature is usually close to its melting point (−1.86 °C), the blood of polar teleost fishes is no more concentrated than their temperate counterparts, and they freeze when cooled to lower temperatures, yet do not freeze in their ocean habitats (247). These polar fish may be in almost constant contact with ice crystals, and ice crystals are present in the gut of some Antarctic fishes; an immune response to ice indicates that ice crystals are likely circulating in the bloodstream as well (567). This phenomenon was investigated in the Arctic by Scholander and colleagues in the 1950s (457), who concluded that the polar fish were supercooled by almost 1°C below their melting point. This paradox was resolved by the discovery that Antarctic notothenid fish (which are over-represented in the Antarctic fauna) produce antifreeze glycoproteins that prevent the growth of ice crystals at temperatures below the melting point of a solution (143).

In general terms, polar fishes are freeze avoiders: slight supercooling is facilitated by the action of antifreeze proteins, which are secreted into the blood, gut and also in the skin epithelia in some species (181). Because temperatures never drop below approximately −1.86°C, and the cell membrane provides an effective barrier to ice propagation, the cells themselves are not generally in danger of freezing directly, and antifreezes do not appear to be intracellular in fish (181). While polar fishes, particularly in the Antarctic, experience a very stenothermal environment, and consequently produce antifreezes year round, fish of northern temperate waters produce antifreezes seasonally, reflecting the seasonal presence of ice in the environment (181).

## Biochemical, Cellular, and Molecular Adaptations to Sub-Zero Temperatures

### *Ice nucleation*

Many low temperature adaptations are common to animals that survive and avoid freezing, and the proximal determinant of the cold tolerance strategy is thus the presence of an efficient ice nucleator. Masking or expulsion of potential ice nucleators is thus key to successful freeze avoidance (155, 627). Many animals overwinter in moist environments, and are readily nucleated by contact with external ice crystals (110). Indeed, some insects appear to require external inoculation to survive freezing (e.g., reference 483). By contrast, other animals may avoid external ice nucleation by the selection of dry hibernacula, the construction of a hydrophobic cocoon, or the secretion of hydrophobic waxes (134,312,499).

The gut of insects is thought to be a significant source of ice nucleators, both through endogenous flora and consumption of exogenous particles and bacteria (610, 614). In many cases, the nucleating activity of the gut contents correlates closely with the SCP of the insect, and appears therefore to be the site of nucleation (614). For this reason, many overwintering insects actively clear material out of their gut prior to the onset of winter (627). Bimodal SCP distributions in Collembola have also been attributed to the presence (high SCPs) or absence (low SCPs) of material in the gut (72, 506).

A number of insects also regulate ice nucleation endogenously. Indeed, this regulation of ice nucleation appears to be the primary difference in the freezing process of freeze-tolerant and freeze-intolerant drosophilid larvae (493). Ice nucleation in overwintering larvae of the goldenrod gallfly *Eurosta solidaginis* appears to occur at calcium phosphate crystals in the Malpighian tubules (381), but most other endogenous ice nucleators appear to be proteinaceous, usually secreted into the hemolymph. Many insects (and also some intertidal gastropods, 231) produce what appears to be endogenous hemolymph ice nucleating proteins (155, 630). In contrast to antifreeze proteins (see below), little is known about the structure and function of these ice nucleators (155, 630). The microbial ice nucleating protein from *Pseudomonas* has a distinct ice-binding site (286), and antibodies raised to this protein cross-react with at least one other ice-nucleating agent (161). The amino acid sequence of a 74 kDa glutamate/glutamine-rich ice nucleating protein from overwintering *Vespula maculata* queens has been determined (157), but has not been subject to further study. Freeze-tolerant larvae of the crane fly *Tipula trivittata* produce a hemolymph lipoprotein that has been more thoroughly characterized (see 159,392). This lipoprotein nucleator appears to consist of two protein subunits and both neutral and polar lipids, and requires a surprisingly high concentration (ca. $10^8$ molecules in 1 μL) to be effective (160). This, and direct scanning-tunneling electron microscopy suggest that the lipoprotein is active only as multiunit aggregates (623).

### *Colligative cryoprotectants*

As mentioned above, the addition of solutes decrease the melting point, and, because of its impact on the interactions between water molecules, the SCP of the solution may be depressed by about twice that amount (628). Because this relationship is independent of the nature of the solute, amino acids, ions, and carbohydrates, and both large and small molecules, will have this effect. A good colligative cryoprotectant will be readily transported through the cell membrane, allowing equal intracellular and extracellular concentrations, be relatively nontoxic, and be easily produced, and able to be consumed at the conclusion of winter.

Both freeze-tolerant and freeze-avoiding insects accumulate carbohydrates in association with increased cold tolerance (313). In general, these molecules are small sugars or sugar alcohols, particularly glycerol, but other molecules, including ethylene glycol, sorbitol, and myo-inositol have been reported. These molecules are usually normal products of carbohydrate metabolism (525). Smaller molecules would be expected to be favored because they represent more colligative effect per glucose input. Glycerol is particularly common, perhaps because of its low toxicity, and has been reported in molar concentrations in a range of species, including Diptera, Collembola, and Coleoptera (314). Accumulation of amino acids, especially proline, has been reported as a cryoprotectant in some insects (e.g., reference 426), but the concentrations do not approach those of carbohydrates. In freeze avoiding insects, high cryoprotectant concentrations depress the SCP through colligative action, and the effect on the SCP is usually slightly greater than that predicted solely from concentration effects on melting points (627).

Carbohydrate cryoprotectants may also improve cold tolerance of chill-susceptible insects. For example, Lee et al. (315) found a slight (ca. 80 mmol/L) increase in hemolymph glycerol associated with rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. Kostal et al. (300) injected a mixture of ribitol and sorbitol into the hemocoel of the bug *Pyrrhochoris apterus*, raising the hemolymph concentration of these two polyols combined to approximately 83 mmol/L and increasing survival of 3 days at −14°C threefold. Overgaard et al. (404) found an increase in glucose content of *D. melanogaster* adults cooled gradually, and suggested that this may account for rapid cold hardening, but an acute exposure that also elicits a rapid cold-hardening response is accompanied by a decrease, rather than an increase, in glucose (341).

Glucose is the primary cryoprotectant in freeze-tolerant vertebrates and earthworms, facilitating osmotic dehydration of the cells (525). Carbohydrate cryoprotectants are also commonly observed in freeze-tolerant insects (140), particularly those species that survive freezing at very low temperatures (e.g. 435, 476). One hypothesis for the cause of mortality in frozen, but intact, cells is that the cell volume drops below a minimum level required to maintain functional integrity (365). By having equal intracellular and extracellular concentrations of a carbohydrate, it is possible that the carbohydrate cryoprotectants increase this minimum cell volume. Carbohydrates, particularly trehalose, are also closely associated with extreme desiccation resistance in anhydrobiosis, where they are thought to protect membrane and protein structure (129). Because the cells in a frozen animal are thought to be in an essentially dehydrated state, it is possible that carbohydrates perform a dehydration-protective role in freeze-tolerant species (525).

### *Noncolligative cryoprotectants*

A hysteresis between the melting and freezing points in a biological solution was first noted in the cryptonephridial fluid of larvae of the mealworm, *Tenebrio molitor,* by Ramsay (428). Since their relationship with ice was elucidated in Antarctic fishes in the 1960s (reviewed in reference 181), agents that cause thermal hysteresis have been reported in many arthropods and fishes (155, 181), as well as in plants, fungi, and other taxa (630). Most thermal hysteresis agents in insects are proteinaceous, while fish thermal hysteresis is associated with proteins or glycoproteins (the latter in cods and notothenids), and there are anomalies that suggest that some thermal hysteresis may have a nonproteinaceous source (see later). The dominant theory of how thermal hysteresis agents prevent the growth of ice crystals in solution is the "adsorption-intereference" theory advanced by Raymond and De Vries (429), the evidence for which is summarized by Fletcher et al. (181). In fishes, the primary function of thermal hysteresis agents appears to be preventing the growth of embryo ice crystals. In freeze-tolerant organisms (including insects and plants), thermal hysteresis agents likely prevent recrystallization of ice once it has formed (297). Thermal hysteresis agents (Fig. 14) are also thought to stabilize highly supercooled fluids (629), and prevent inoculative freezing in insects in contact with external ice (193). Two groups have reported the expression of antifreeze proteins in *D. melanogaster*. The expression of AFPs from the beetle *Dendroides canadensis* or the spruce

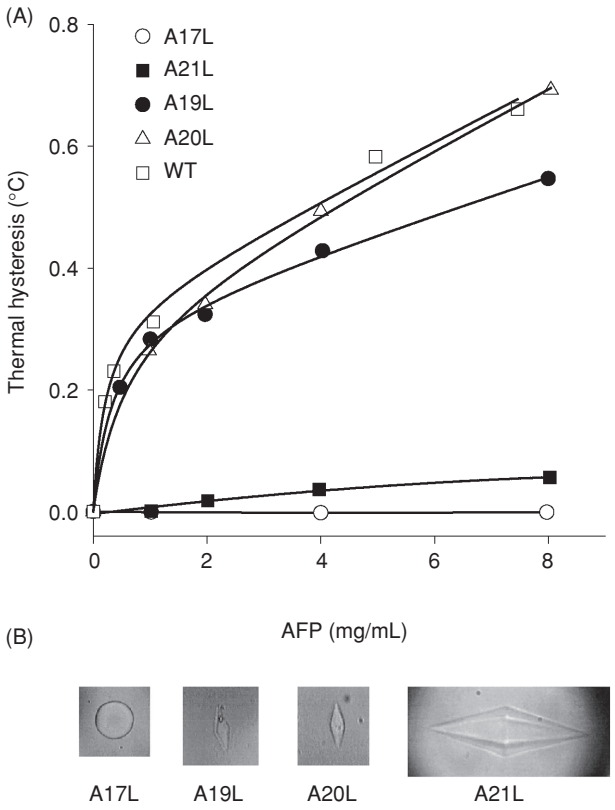


**Figure 14** Thermal hysteresis (°C difference between melting and freezing temperatures) as a function of antifreeze protein concentration (A) for synthetic Type I AFP and variants with Ala residues replaced with Leu. (B) The morphology of the ice crystals for four of the different variants. Adapted, with permission, from reference 22.

budworm *Choristoneura fumiferana* in *Drosophila* results in thermal hysteresis activity and also an improvement in survival of chilling at 0 and 4°C, but does not appear to improve tolerance to cold shock (330, 395, 561).

Antifreeze proteins and glycoproteins generally have repetitive, hydrophilic elements that match the structure of water molecules in ice crystals (181). In insects, fewer AFPs have been studied in depth. Beetle AFPs that have been sequenced appear to be closely related (211, 424), but differ significantly from those of Lepidoptera (152) or a springtail (208). In fishes, there are four main types of antifreeze proteins (types I-IV) and antifreeze glycoproteins (AFGPs). AFGPs are made up of a variable number of repeats of an Ala-Ala-Thr motif bound to a disaccharide (galactosyl-*N*-acetylgalactosamine) at the hydroxyl group of each Thr (181). The fish AFPs show a large diversity, ranging from helical proteins (Type I) to globular (Type II); see Fletcher et al. (181) for a full description of their diversity and properties.

Antifreeze proteins in insect hemolymph are hyperactive compared to fish antifreezes (209). One reason for this may be the presence of other molecules that enhance the activity (158). Glycerol, citric acid, and a thaumatin-like protein have all been implicated as enhancers of both thermal hysteresis and the ability of AFPs to prevent ice nucleation (156,583). In addition, antifreeze proteins themselves seem to bind to one another. This results in an enhancement of activity, possibly because the AFP+enhancer protein blocks a larger surface of the ice crystal, preventing growth (582).

There are a number of anomalies in the noncolligative cryoprotection literature, for example, agents that inhibit recrystallization, but do not impart thermal hysteresis (592). The arctic tenebrionid beetle *Upis ceramboides* has a nonproteinaceous thermal hysteresis agent that appears to be a xylomannan glycolipid, built around a disaccharide core (579). The sugar component of this glycolipid is distinct from the saccharide component of antifreeze glycoproteins found in fish (579). The function, synthesis, and prevalence of this antifreeze glycolipid remain to be determined.

### *Aquaporins and freeze tolerance*

The general model of extracellular freezing in both invertebrates and vertebrates relies upon the rapid exit of water from cells to maintain osmotic equilibrium (497), and also the equilibration of carbohydrate cryoprotectants between the interior and exterior of the cell. Aquaporins (AQPs) and aquaglyceroporins (AQGP) are transmembrane proteins that form channels that allow the bulk movement of water and (in the case of AQGPs) small uncharged solutes in and out of cells (293). Izumi et al. (268) used mercuric chloride to inhibit water transport by AQPs, and therefore freeze tolerance, in the rice stem borer, *Chilo suppressalis*. Philip and colleagues (412, 413) showed that AQPs increase in abundance in association with the seasonal acquisition of freeze tolerance in larvae of the gallfly *Eurosta solidaginis*, and that $HgCl_2$ inhibited freeze tolerance in mid-gut and fat body cells, but not in salivary gland. Whether AQPs of freeze-tolerant species are distinctive for their structure and function, or are a case of simple transcriptional regulation remains to be determined, as does their role in vertebrate freeze tolerance.

### *Genes associated with subzero temperatures*

Molecular studies have revealed a number of genes whose expression is associated with cold exposure (reviewed by (90, 526). Although many of these studies have been in *D. melanogaster*, reflecting the genomic tools available in that organism, nonmodel species, including Collembola (89) and frogs (527) have been studied using a cross species or EST-library approach. In freeze-tolerant wood frogs, transcription factors appear to respond to freezing stress, leading to other, downstream, responses associated with stress metabolism and avoiding cellular damage from hypoxia and osmotic stress (527). In frogs, the gene expression responses to freezing appear to be organ-specific. For example, the ribosomal phosphoprotein P0 is upregulated in the brain but not the liver in frozen animals versus unfrozen controls (616), while other genes (e.g., references 47 and 364) are expressed only in the liver. In insects, genes that respond to cold stress include known stress proteins, like the heat-shock proteins but also cytoskeletal and metabolic genes. The enigmatic gene *Frost*, which appears to code for a secreted mucin-like protein (206), has received particular attention in *D. melanogaster*, having been identified as a candidate in several studies (e.g., references 375, 425, and 544). *Frost* inhibition increases chill coma recovery time (96), but its role in survival of acute and chronic cold exposure has not been elucidated. However, for most insects, it is apparent that gene expression occurs during recovery from cold exposure, rather than during the cold exposure itself (494).

## Evolution of subzero temperature tolerance

### *Evolution of cold tolerance and AFGPs in fishes*

Antifreeze proteins and glycoproteins have arisen on multiple independent occasions in the teleost fishes. Type I and II AFPs are present in multiple different clades, and antifreeze glycoproteins have evolved independently in Antarctic notothenioids and Arctic cod (82). Antifreeze glycoproteins in the Antarctic notothenioids derived from a pancreatic enzyme (trypsinogen) approximately 14 Ma, a timing that is concordant with the estimated first appearance of ice in the Antarctic oceans, and also reflective of the divergence times of the Antarctic notothenioids, all of which carry the trypsinogen-derived AFGP (83). By contrast, the Arctic cod AFGP, which shares many of the same structural elements as the notothenioid glycoprotein, including the same disaccharide, has a more recent origin, probably from a different ancestral gene (82). Type II AFPs in northern fishes exhibit an even more striking convergent evolution: these AFPs have evolved on multiple, independent, occasions from the same C-lectin precursor (181). Like the AFGPs in the notothenioids, the Type

III AFP in zoarcids (the only group with this type) appears to be derived from a common sialic acid synthase progenitor (21). Precisely how this group came to have AFP-carrying members in both polar oceans remains to be explained (181). Up to 150 copies of AFP-encoding genes have been reported in fish. Fletcher et al. (181) have suggested that this extraordinary proclivity for multiplication is a consequence of the extremely strong selection for subzero temperature tolerance, and point out that multiplication is also common in other systems where a single gene that may confer a significant survival benefit in the face of an environmental stressor, for example in genes associated with xenobiotic resistance in insects (379).

### *Acquisition of antifreeze proteins by horizontal gene transfer*

By contrast, the most parsimonious origin of the AFP detected in the sea ice copepod *S. longipes* is that it was transferred horizontally from a sea ice diatom (291). Kiko (291) demonstrates that the gene is expressed ubiquitously in the copepod, which suggests that the AFP is not acquired from the brine channel or from ingested diatoms, and rules out the likelihood of convergent evolution. Kiko (291) proposes that thermal and salinity fluctuations in the brine channels of growing sea ice may be analogous to the transformation conditions used in laboratory molecular biology. It remains to be seen whether these brine channels harbor a higher than expected rate of horizontal gene transfer in other genes and taxa. Horizontal gene transfer has also been proposed to explain the occurrence of closely related Type II antifreeze proteins in distantly related herring and smelt in the northern hemisphere (210).

### *Evolution of insect cold tolerance strategies*

Freeze tolerance is found in at least six orders of insects, and even within orders has probably arisen multiple times as insects have invaded cold environments (86). Although freeze avoidance has been suggested as the ancestral state (569), parsimony and evidence from *Drosophila* suggest that ancestral insects were probably chill susceptible, and that freeze avoidance has also arisen on multiple occasions (530).

The selective pressures that lead to insects adopting freeze tolerance or freeze avoidance have been subject to debate. It is clear that there are some constraints that predispose insects towards one strategy or another, for example, the theoretical lower limit to supercooling means that most insects exposed to ambient temperatures in the high arctic are expected to be freeze tolerant (29), although exceptions may occur (476). Similarly, freeze tolerance may be an advantage for insects in environments rich in ice nucleators (for example, very moist habitats), where avoiding freezing would be all but impossible. In more temperate environments, the advantages of one strategy or another are unclear. Sinclair and colleagues (491,492) have suggested that there is a greater-than-expected proportion of freeze-tolerant species in the southern hemisphere because freeze tolerance allows insects to survive unexpected cold snaps in the summer without a requirement to clear food and ice nucleators out of the gut. There are also energetic and water balance advantages to freezing, as compared to spending the winter in a supercooled state (265,266), although these may be offset by the (poorly understood) costs of repeated freezing and thawing (86).

### *Evolutionary pressures leading to freeze tolerance in terrestrial vertebrates*

Freeze tolerance has evolved independently in several groups of vertebrates, including lizards, frogs, and turtles (528). Inoculation by external ice appears to be the primary cause of ice nucleation, and thus may determine the use of freeze tolerance (110). However, in many cases, habitats where freeze-tolerant vertebrates are found also include aquatic environments where closely related species may overwinter without risk of freezing (543). Although the freezing process in vertebrates is likely energetically expensive due to the rapid mobilization of glucose, there may still be significant energetic savings associated with the terrestrial habitats (and to being frozen). Voituron et al. (574), using data primarily from vertebrates, constructed a model of the relative advantages and disadvantages of freeze tolerance *versus* supercooling, finding that the energetic status and cost of freezing, combined with the ability of the organism to withstand repeated or sustained freezing, are likely the primary factors determining the evolution of one strategy or the other.

## Behavioral Control over Body Temperature in Ectotherms

One of the most interesting traits of many ectotherms is their ability to thermoregulate when provided a choice of temperatures. Most ectotherms do not possess much capacity to elevate or maintain high $T_b$ through the metabolic processes utilized by endotherms. Instead, ectotherms are completely dependent on obtaining heat from the environment to maintain $T_b$ (468,500). Sustaining high $T_b$ is conceivably the most important variable affecting performance in ectotherms, since temperature has profound effects on almost all physiological aspects of life. Environmental temperature is not the only determinant of $T_b$; the environment itself strongly influences the rate with which heat is added or withdrawn from the body. Water is an extremely effective thermal conductor, especially with mixing. Indeed, the thermal conductance of water is approximately 25 times that of still air, meaning that although many aquatic ectotherms react to, and even select specific thermal habitats (601), it is within the terrestrial ectotherms that some degree of internal thermal stability can derive from careful behavioral selection of temperature. Even so, animals with significant water loss (such as amphibians and terrestrial molluscs) exhibit a high degree of evaporative cooling that can prevent them from achieving $T_b$ above environmental temperatures (263). Therefore, we will focus most of our examples

on reptiles, since their evolutionary history has stronger ties to terrestrial environments and, thus, they are "emancipated" from the influences of water on their thermal biology. The result is that temperature selection and regulatory patterns are more effective in animals that can rely on the lower thermal conductivity of air allowing for greater thermal inertia, and thus some maintenance or temporal control over $T_b$.

Reptiles (namely, crocodiles and lizards, although numerous snakes may exhibit similar behaviors) regulate their $T_b$ by utilizing behavioral mechanisms thereby, allowing them to maintain a preferred $T_b$ (31,32,45,53,115,259,295,385,540), often considered to be within a range of temperatures which correspond to their physiological optimum (7). Reptiles maintain and regulate $T_b$ behaviorally by a dual set-point thermoregulatory mechanism rather than precise control around one particular $T_b$ (32, 295). A single set-point thermoregulatory mechanism would be when a reptile defends a particular optimal $T_b$ (295), whereas dual set-point thermoregulatory mechanism can be described by the avoidance of $T_b$'s above an upper temperature threshold (upper set point), as well as $T_b$'s below a particular low temperature threshold (lower set point) (32, 295). The area between the upper and lower temperature thresholds is often termed the nonthermoregulatory zone, although this is misleading since animals will voluntarily seek to find these temperatures (69, 70). However, when the reptile's $T_b$ is within this nonthermoregulatory zone, the "drive" to seek different temperatures is decreased and the reptile is unrestrained to engage in other activities (31, 237).

There are a number of options to gain heat from the surrounding environment when $T_b$ is below the preferred $T_b$. One of the most important ways to obtain heat from the environment is from solar radiation; animals making use of basking (Fig. 15) to warm up are referred to as heliotherms. Those that make use of heat transfer through direct contact with the substrate that has been heated by the sun are called thigmotherms. One way to exploit solar radiation is through postural and positional adjustments. For example, when lizards are outside their preferred $T_b$, they orientate their body perpendicular to the sun's rays and spreads its rib cage to maximize surface area exposed to the sun (53). When the lizard has increased its $T_b$ to its preferred level the lizard can now change its position so that its head faces the sun thus becoming parallel with the sun's rays (53). Being parallel to the sun's rays, in combination with decreasing the surface area exposed to the sun by relaxing the rib cage ultimately minimizes the exposure to the sun's radiant heat, and slows down further rises in $T_b$ (53).

Probably the most salient behavioral mode of thermoregulation is shuttling (153), which has been studied primarily in lizards (70) and crocodiles (470, 471). Cowles and Bogert (115) initially described shuttling in the field as a heat-seeking and heat-avoidance behavior by a reptile to regulate and maintain a preferred $T_b$ (31,32). This behavior can be demonstrated in a laboratory setting where a thermal "choice" device is set up with one side being cold and another side hot. Upon entering the hot side, the reptile's $T_b$ is assumed to be below the preferred level. Body temperature then rises to its preferred level; however, $T_b$ will continue to rise and may exceed lethal levels as long as the lizard remains on the hot side. Once the lizard's $T_b$ surpasses it preferred level, a heat avoidance behavior occurs. The lizard will quickly search out a cooler environment resulting in a repositioning to the cooler side of the box. Once again, the lizard will stay in the cold side until it cools below its preferred $T_b$, then a heat-seeking behavior will occur and the lizard removes itself from the cold side. This experiment simulates similar environmental challenges like fluctuating temperatures or shuttling between sun and shade throughout the day. Interestingly, however, similar shuttling behavior can be observed in fish (411, 459, 516) and crayfish (337) in laboratory settings and serves as a convenient means to assess voluntary thermal preference. However, one caveat regarding behavioral thermoregulation is that it can

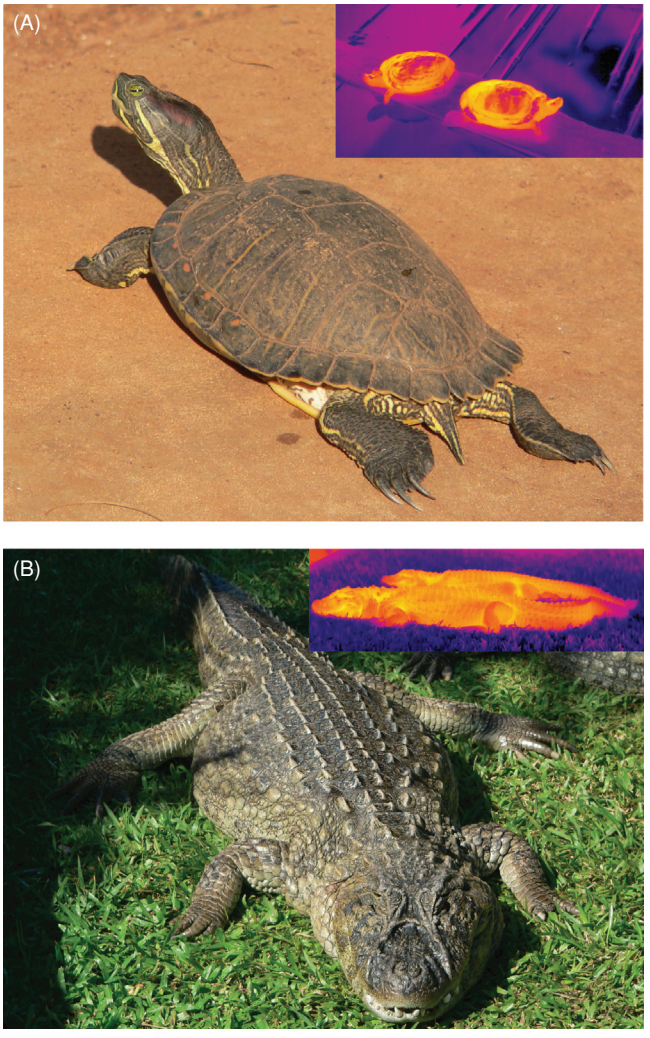


**Figure 15** Behavioral thermoregulation in ectotherms, demonstrated by basking. (A) *Trachemys scripta* with outstretched limbs (inset diagram is a thermal image showing two individuals of the same species). (B) *Caiman latirostris* exposed to full sun reaches temperatures well above ambient temperature (inset diagram is a thermal image showing two individuals of the same species). Images courtesy of G.J. Tattersall.

eliminate the need for other thermoeffectors. For example, lizards, when placed into thermal gradients will not exhibit shuttling behavior (69, 70, 602), but still manage to maintain similar body temperatures. Indeed, although ectotherms can be induced to exhibit shuttling, when shuttling becomes more costly than alternative means, there can be consequences for the precision of thermoregulation (69, 70).

However, behavioral responses are inherently physiological responses, since the simple evocation of muscular responses to effect movement involves neuromuscular coordination. As well, the ability to sense temperature is a critical neurophysiological response required for behavioral thermoregulation. Relatively little data exists on the functional role and location of molecular temperature sensor in ectotherms relevant to normal thermoregulation, although peripheral cold and warm sensation has been described and characterized in numerous ectothermic vertebrates (121, 122, 124, 512-514, 545, 608). Recent evidence for the involvement of temperature-sensitive ion channels (thermoTRPS) in the thermoregulatory behavior of crocodiles was discovered using systematic injections of antogonists of TRPM8 and TRPV1; blocking these ion channels virtually eliminates all thermoregulatory shuttling behavior (469), although the location of these sensory responses and their distribution and importance throughout the body are unknown. Thermal sensory information is relayed to central control centers, namely, within the hypothalamus (which itself contains thermosensitive neurons), and ascending pathways project to cortical regions where behavioral choices are evoked. Although little recent work has examined the central control mechanisms, it is apparent that reptiles exhibit both peripheral and central thermosensation (545), and that the hypothalamus plays a vital role in integrating all the thermoregulatory behaviors, since hypothalamic lesions abolish the tendency the normal thermal preferences, and animals behave as if they cannot sense environmental temperatures (391).

## Physiological Control over Body Temperature in Ectotherms

As discussed previously, ectotherms (namely, reptiles) regulate their $T_b$ primarily by behavioral mechanisms such as postural and positional adjustments as well as thermal shuttling. For these behavioral mechanisms to be efficient and effective, however, physiological contributions to body temperature also occur.

### Facultative endothermy in ectotherms

In extreme cases, such as what is observed in numerous tuna species, a morphological arrangement of blood vessels in the form of a rete allows for heat produced in the active swimming muscles to be retained. Unlike most fish, the arteries feeding the tuna's swimming muscles originate from peripheral beds, and run parallel to veins that drain the internal musculature. The morphological arrangement capitalizes on the natural heat produced from normal muscle metabolism (519-522). Similar systems have evolved within the billfish, except that the heat is retain primarily within the eye and the brain. The eyes of these fish possess a heater organ, which is essentially a derived muscle tissue lacking myofibrils, but consisting primarily of sarcoplasmic reticulum. The futile cycling of calcium ions through ryanodine receptors is coupled to the $Ca^{2+}$ ATPase activity also located in the sarcoplasmic reticulum. The excess ATP consumption is coupled to elevated levels of metabolism, raising the heat production inside the heater organ (378).

However, "global" heat production of significant magnitude is unusual in most ectotherms; nevertheless, some exceptions do exist. For example, numerous insects exhibit facultative and regional endothermy, with heat production from the flight muscles raising thorax temperature substantially above ambient temperature (41, 54, 227, 228, 357, 563). In a few cases, these insects are capable of maintaining nearly constant thorax temperature across a wide range of ambient temperatures, underlying the importance of muscle temperature for fueling flight. However, in addition to flight, brood thermoregulation and defense are other examples of insect thermoregulation, particularly within the social insects (239). In vertebrate ectotherms, there are relatively few instances of facultative endothermy, although the best known examples occur in snakes. Facultative maternal brooding has been observed in numerous species of pythons (226,485). By adopting a muscular-based thermogenesis, female pythons are capable of raising and maintaining elevated brood temperatures ($\sim$10-20°C increase above ambient). This response has recently been proposed to be adaptive, not only in terms of augmenting the rate of incubation, also in terms of physiological performance of the hatchlings (485). Nonmuscular-based facultative endothermy appears to produce a lower magnitude thermogenic response ($\sim$1-4°C increase above ambient); the postprandial increase in metabolism in numerous snake species can lead to metabolic rates rising by 10 to 40 times above basal levels (5,461,551), which leads, likely passively, to a prolonged rise in body temperature (542).

### Cardiovascular control over body temperature in ectotherms

In pioneering studies on the physiology of thermoregulation in lizards, Cowles (114) demonstrated that the desert iguana's $T_b$ warmed much faster than it cooled, and that the desert iguana exhibited a higher blood flow to the periphery (skin) during warming. Bartholomew and Tucker (37) validated Cowle's (114) suggestions of physiological thermoregulation by finding that the bearded dragon also followed the same trend as the desert iguana $T_b$, whereby it heated faster than it cooled. Bearded dragons heated 1.3 times faster than they cooled (37); furthermore, both heating and cooling rates were faster in live animals than in dead bearded dragons, and the dead bearded dragons heated and cooled at almost identical rates (214,216).

These investigations suggest that reptiles must have the ability to actively control heat transfer between their body core and the environment (462). Having the ability to control the rate of heating and cooling allows for rapid absorption of heat during basking, therefore, decreases the amount of time taken to achieve preferred $T_b$ (37, 466, 467, 469). Similarly, during cooling in shaded areas or nesting sites, the reduction of cooling rates decreases the rate of heat loss, thereby lessening the time spent basking later on in the day or when the reptile emerges from its nest site in the morning (37, 462, 466, 467). This difference between the heating and cooling rates of reptiles has been termed "thermal hysteresis" (note the distinction from the "thermal hysteresis" of freezing and melting points associated with antifreeze proteins) (Fig. 16).

The differences among the heating and cooling rates in reptiles are brought about by cardiovascular alterations, where blood flow to or away from the periphery will increase or decrease thermal conductance between the body core and the surrounding environment (36, 37, 164, 216, 377). It has been speculated that an increase in blood flow to the periphery results in an increase in thermal conductance resulting in an increase in heating rate (36, 37, 163, 164). Conversely, when blood flow to the periphery is reduced there is a decrease in thermal conductance leading to a slow rate of cooling (163, 164, 377). Experimental studies also show that increased movement of blood to the periphery causes an increase in heart rate (36, 37, 214). The heart rate pattern has been documented in various reptile species demonstrating that during the heating process there is a rapid increase in heart rate and oppositely during the cooling process there is a reduction in heart rate. Therefore, at any particular $T_b$, heart rate is faster during heating than during cooling. This phenomenon of differences between heart rate during heating and cooling has been termed "heart rate hysteresis" (37, 214, 377, 466); however, the basis for the physiological control is not the cardiovascular regulation of heart rate, *per se*. Blocking autonomic control of heart rate does not block the peripheral blood flow responses, nor the changes in rates of heating and cooling (469), suggesting that the control of heart rate itself is not the active mechanism producing peripheral blood flow changes. Indeed, prostaglandins have been found to be involved in the control of peripheral vasomotor activity in bearded dragons during heating and cooling (467), suggesting the responses driving the association between thermoregulation and cardiovascular control are based on peripheral control.

The Galapagos marine iguana is a classic textbook example that illustrates how augmentation to the cardiovascular system can be used to maintain and regulate preferred $T_b$'s. The marine iguana's preferred $T_b$ is roughly 37°C; however to obtain food they must undertake in prolonged dives into the sea that has a temperature ranging from 22 to 27°C (36). When submerged in the sea at cold temperatures the only means that the marine iguana has to control the rate of cooling is by changes in blood flow. Therefore, to reduce the speed of cooling and to optimize the amount of time spent foraging in the water there is a vasoconstriction of peripheral blood vessels leading to the reduction in cutaneous blood flow followed by a decrease in heart rate. This results in a decline in thermal conductivity between the warm inner core and the cool surfaces of the marine iguana. This alteration to blood flow significantly reduces the rate of cooling when submerged in the sea. When the marine iguana leaves the sea after foraging it uses postural changes to behaviorally control its warming back to its preferred level (36). However, to reduce the time taken to achieve preferred $T_b$ and to accelerate the rate of heating there is a vasodilation of peripheral blood vessels that results in an increase in cutaneous blood flow. This increase in peripheral blood flow is followed by an increase in heart

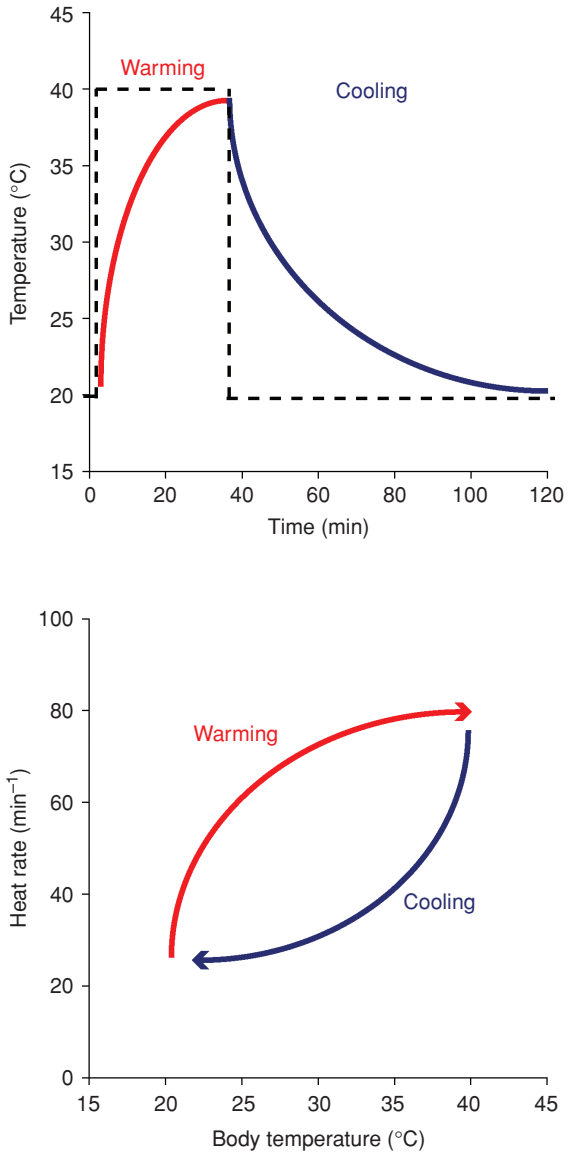


**Figure 16** Representative schematic of the asymmetry in heating and cooling rates (thermal hysteresis) in many reptiles (A) manifests from a differential distribution of blood flow to the periphery during heating compared to cooling, resulting in a hysteresis in the heart rate (B) response to changes in body temperature.

rate amplifying the thermal conductivity with the cooler inner core and the warm surfaces of the marine iguana.

One caveat needs to be made regarding the notion of cardiovascular control regulating heat exchange in reptiles. Firstly, it was argued by Langille and Crisp (310) that the thermal and heart rate hysteresis response could be a simple physical manifestation of more rapid effects of temperature on peripheral blood viscosity altering the relationship between flow and pressure in smaller vessels. Warm skin would have lower viscosity blood, which would lead to higher peripheral blood flow, and faster heat exchange rates, but not necessarily due to an autonomic/physiological regulation associated with thermoregulation. To compound the issue, a heart rate hysteresis has also been observed in crayfish (207), a small aquatic animal, which cannot obtain the same degree of thermal benefit from differentially directing flow to the periphery. Obviously, the peripheral dilation that occurs in an aquatic animal that cannot bask would not substantially improve rates of heating, so understanding the role of temperature control on the evolution of the cardiovascular system will require further research. Finally, Crawshaw (123) demonstrated that brain temperature changes much faster than deep core temperature in fish. As brain temperature is seldom measured in studies of ectotherms there are few assessments in the scientific literature of whether the more rapid changes in central thermosensors in the brain (68, 222) drive the apparent hysteresis in cardiovascular control.

## High-temperature adaptations in ectotherms

### *Modifying radiative heat loads: Temperature effects on skin reflectance*

A common response within the vertebrates to changes in temperature (over multiple timescales) is a change in skin reflectance, typically in the visual spectrum, and thus manifesting in a change in color. On the surface, the alteration in skin reflectance will aid in heat absorption during postural and positional changes. A multitude of studies in ectotherms have been performed, primarily in lizards, on this particular phenomenon, where it has been shown that when the lizard is cool or below it is preferred $T_b$ it adopts a darker skin coloration (53, 114, 115). This darkening achieves maximum absorption of radiant heat and allows the lizard to reach its preferred $T_b$ more rapidly. Once preferred $T_b$ has been reached or slightly surpassed, the skin lightens (53, 115). When the skin lightens, it becomes more reflective of the sun's radiant heat, slowing down or abating further heating (538). Being able to control the reflectivity and absorption of the sun's rays is a mode of thermoregulation that is available to many reptiles and amphibians (Fig. 17), and these rapid and reversible skin color changes have been observed in many species of reptiles and amphibians exposed to altered light and incident radiation levels (74, 87, 95, 294, 481, 581).

Skin coloration changes have been defined in two ways: morphological color change and physiological color change.

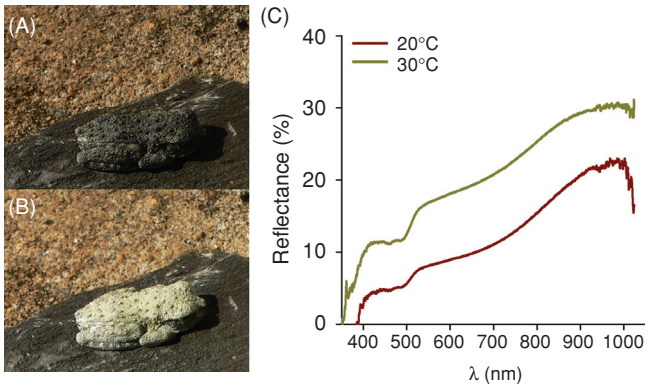


**Figure 17** Temperature-related skin reflectance changes in the Brazilian frog (*Bokermannohyla alvarengai*) at 20 (A) and 30°C (B) Corresponding changes in skin reflectance are shown in (C) (data adapted, with permission, from reference 538).

Morphological color change is induced slowly, taking days and even months to develop, through the development of new structures within the pigment cells, while physiological color change is induced by the rapid movement of organelles within cells or an ultrastructural rearrangement that can take place within minutes (479). Physiological color change in response to environmental stimuli, such as changes in background color, light intensity, or temperature, has been documented in many reptilian and amphibian species (339). Skin color change in response to background serves as a means of protection against the detection of visual predators searching for prey (294). More importantly in terms of thermoregulation the skin color can also play a significant role in the rate of heat transfer between the body core and the environment (294) especially since amphibians or reptiles with dark skin absorb more incident radiation (74). Numerous species of reptiles (including crocodiles and lizards) and amphibians (mainly frogs) have been shown to exhibit changes in skin coloration during basking or cooling (408).

Skin reflectance change can over multiple time scales. Nearly instantaneous responses occur within minutes, reflecting intracellular processes that alter pigment spatial migration (see later). Slower changes can occur over longer time scales, reflecting acclimation or background adaptation (518). Over evolutionary time scales, skin reflectance may also change, which has led to the thermal melanism hypothesis, which proposes that darker colored individuals are at an advantage under cool climates. Interestingly, the thermal melanism hypothesis is slightly at odds with an older, lesser known observation, referred to as Gloger's rule, which suggests that cool, dry climates favors lighter coloration in animals, although this earlier observation utilized a non-systematic approach (see reference 568), and was possibly confounded by skin color reflecting selection for camouflage or crypsis, rather than temperature. Support for the thermal melanism hypothesis exists in insects (animals with no apparent physiological color change) and in lizards which undergo rapid and reversible changes in skin reflectance (91-93);

however, caution should be exercised in interpreting studies obtained from animals that also undergo rapid color changes, since this "reaction norm" to temperature needs first to be established within each species before comparing among species inhabiting different thermal regimes and inferring broad scale adaptive patterns in skin reflectance.

Several studies have been conducted to establish that skin color change has a thermoregulatory function. Demonstrating a change in reflectance itself does not itself imply a change in internal temperature; however, the speed with which skin color changes in response to fluctuating ambient temperatures suggests a purpose in thermoregulation (119). Furthermore, low reflectance (high absorbance) of short-wave visible light causes small reptiles and amphibians to maximize heat gain until they reach a critical $T_b$, above which the skin lightens or "blanches" so much that it reduces further radiant heat gain (479-481). They absorb short wavelength radiation from the sun and exchange long wavelength radiation with their environment (34). In some species, this effect is so extreme that the skin adopts a shiny, but chalky appearance (290, 538). Fieldwork has confirmed these physical predictions. Pearson (408) established the importance of skin darkening in the thermoregulation of high altitude lizards and demonstrated the lizards' capacity for controlling the absorption of sunlight by changing skin coloration. He was able to quantify the change of reflectance that occurs as the lizards warmed (i.e., low reflectance when cold/high reflectance when warm) and thus determined that controlling the rate of absorption of radiant energy can be considered an adaptive mechanism geared toward the maintenance of body temperature. Comparable results have been obtained in amphibians (74, 538).

Body size in ectotherms may play a role in the expression of skin reflectance changes. Sherbrooke (479) demonstrated a broad range of reflectance changes in response to temperature changes in several species of *Phrynosoma* lizards of diverse sizes. He determined the skin-darkening values using skin studied *in vitro* and concluded that the smaller species, *Phrynosoma modestum* exhibits the broadest range of reflectance changes in comparison to the larger species *P. cornutum* and *P. solare*. The latter two species were placed on a black-and-white background and failed to match to the background, thus suggesting that color changes in the species that occurs in the wild were likely associated with thermoregulatory needs, rather than crypsis; cool lizards darken early in the day, thereby increasing solar thermal gain, whereas warm lizards lighten at midday to reduce thermal gain and avoid overheating. Rapid color changes are more effective in animals with less mass, such as smaller reptilian species, *P. modestum,* with an adult body weight under 10 g (479). This species exhibits the broadest range of color (and therefore, reflectance) changes in response to changing temperatures, due to its larger body-surface area/volume ratio (479). Overall, the ability to regulate temperature in any reptile via changing body color is a direct result of their ability of changing solar absorption at the skin (518). Given the small size of most amphibians, temperature-induced reflectance changes may play significant roles in temperature regulation.

Indeed, this has been confirmed for some amphibians. King et al. (294) also inferred that color changes have an important role in thermoregulation, especially in amphibians such as green tree frogs, *Hyla cinerea*. They were able to show a high capacity for physiological skin color change in this species by fluctuating background brightness and temperature. Tree frogs exhibited higher brightness at higher temperatures but did not match background brightness, exposing a background-by-temperature interaction. The skin of amphibians may constrain the utility of color change as this organ is also of importance for gas exchange and water balance, although previous studies have postulated that "waterproof" frogs that spend time exposed to solar radiation benefit more from skin reflectance changes than more shade dwelling, water-dependent species (294, 510, 538, 553). Reptiles, in general, exhibit much lower rates of cutaneous water loss than amphibians (488), suggesting that they are capable of capitalizing on the thermal benefits of basking without the osmotic costs of exposure. It is expected, therefore, that reptiles would exhibit substantial changes in skin color (i.e., reflectivity) to avoid overheating as temperature rises.

### *Properties of the dermal pigment cells*

Changes in skin color or color patterns in ectotherms are ultimately due to the motile activities of specialized pigment cells called chromatophores, located in the dermis (403). The rapid physiological coloration change is promoted by the reversible and bidirectional translocation of pigment granules within chromatophores in response to environmental stimuli (24). During a physiological color change, information on relative light levels in the environment enters the animal's eyes, influencing the neural and endocrine systems. The eyes play an essential role in the background adjustment responses by comparing the amount of light falling directly on the eye to that which reaches the eye indirectly following reflection from the background (25). A primary color response is one in which chromatophores, respond directly to the environmental stimuli by simultaneous movements of guanine crystals located within the cell (403). The primary system of skin color change has no known neural connection; therefore full details on the neurophysiological thermoregulatory connections to skin reflectance have not yet been discovered.

Chromatophores themselves can be divided into three types according to their pigment color and internal structures: melanophores, xanthophores, and iridophores (25). Ultimately, rapid changes of color depend entirely on the changes of these dermal pigment cells, their architectural arrangements, and their occurrence in the skin (479). Xanthophores are outermost in location, typically possess a yellow color, and are derived from carotenoids and pteridins (25). The role of xanthophores in the overall color of the skin is mainly in their involvement in establishing color patterns; for

example, they are responsible for the red spots of the red-spotted newt (*Notophthlamus viridescens*). The reddish spots in this species are noticeable when the pigment granules, pteridines, and carotenoids, are dispersed within the peripheral margins of the cell (25). Xanthophores are not normally involved in physiological color changes (403).

Iridophores are cells that contain pigment stored into thin, flat microplatelets and are located immediately beneath the xanthophores. They are considered to be the reflecting cells that contain a combination of crystals of guanine, hypoxanthine, or adenine (219), oriented in such a way as to reflect light efficiently (25). The principal pigments of iridophores are purines, of which guanine is the most abundant. Iridophore pigment is further enhanced when dispersed to the peripheral margins of the cell and appears silvery or golden; otherwise the pigment appears opaque when concentrated toward the center of the cell (25). Iridophores are confined to the dermis of the animals; when the melanin is dispersed their reflecting capacity is heavily obscured (403).

Melanophores, as their name implies, possess black or brown granules of melanin, contained in ultrastructural units called melanosomes. Melanosomes play the most dominant role in rapid changes of coloration (25). Skin coloration changes are mediated via the dispersion (darkening process) or the aggregation (lightening process) of the melanosomes within melanophores. Epidermal melanophores are found just above the germinative layer of the epidermis where they form finger-like structures that extend between adjacent epidermal cells that play a significant role in morphological color changes (294). Dermal melanophores occur in greater numbers in the dorsal integument, which leads to the lighter coloration common to the ventral surface of many reptiles and amphibians (25), but presumably more important role in thermoregulation. In the dermis, they form finger-like structures or dendrites that extend between the iridophores and xanthophores. These dendrites are responsible for the deposition of melanin granules in epidermal cells thus allowing for physiological skin color changes. Prolonged hormonal stimulation of melanophores can be triggered by environmental stimuli such as temperature or background color, resulting in the accumulation of melanin, and consequently darkening the dorsal skin of the animal (344). In some species such as *Agalychnis dachnicolor* there appears to be one melanophore for every iridophore (25). However, probably the most important characteristic of melanophores is that they are capable of obscuring the reflecting capability of the brightly colored pigments within the iridophores (344), since light that would otherwise be reflected by the iridophores is instead absorbed by the dispersed melanin pigment.

When the melanosomes aggregate around the melanophore cell nucleus, away from the dendrites, the skin lightens. Skin darkening is the result of the dispersion of melanosomes into the dendrites between the melanophores and xanthophores. The dendrites are static and there is no movement or interaction between them, thus allowing the melanosomes, or pigment granules, to be free to migrate in and out of the dendrites without obstruction from either xanthophores or iridophores (25). The dispersion of melanosomes within melanophores will overrule any reflectance effects within the iridophore layer, as well as dispersion of pteridines and carotenoids within xanthophores (25). It is of special interest to mention that the skin of reptiles such as *Anolis carolinensis* has no sympathetic innervation, which suggests that color change must be controlled by the binding of circulating adrenergic hormones (566), such as epinephrine, norepinephrine, and melanotropin (MSH). A change in skin coloration is the direct reaction to the activity of these hormones at receptors in the membranes of the chromatophores (213).

A number of endocrine glands are responsible for releasing hormones which have a direct effect on chromatophores either by inhibiting or promoting the release of melanin within the dendrites and the mobilization of the melanosomes (339). Granule migration, which promotes skin darkening, is controlled by rising levels of circulating α-melanotropin (MSH), which is secreted from the pars intermedia of the pituitary gland. Other mechanisms of rapid color change within melanophores rely on receptors located within the melanophores (479). These respond to circulating catecholamines which, when released, act as potent effectors through α-adrenoreceptors that lighten skin color and β-adrenoreceptors that darken skin color (25). Catecholamines may also directly inhibit MSH release from the pars intermedia (220). For example, epinephrine is known to promote a slow darkening of the skin, but is not as effective as α-MSH, which is capable of stimulating the chromatophores within minutes (566). In addition, norepinephrine will override β-adrenoreceptor stimulation via epinephrine, and invert the darkening process and lighten skin coloration. Isoproterenol, an adrenoreceptor agonist, will promote skin darkening through β-adrenoreceptor activation in melanophores (479), suggesting that β-adrenoreceptors play an active role in melanosome migration. The effect of stimulating α-adenoreceptors will have a greater impact than the effect of β-adrenoreceptors in the presence of any α-stimulating hormones (479).

In terms of potential thermoregulatory relevance, the skin-darkening responses to a given concentration of MSH vary when held at different temperatures, indicating that melanophore sensitivity changes in response to temperature fluctuations (480). Furthermore, the melanosome migration response is sensitive to catecholamines; propanonol (a β-adrenergic antagonist) can slow down the chromatophore response to α-MSH, as well as completely inhibiting the chromatophore response to epinephrine in species such as *A. carolinensis*. This response will affect the duration of darkening in the skin and can completely block skin color change (566). Lowering the affinity of pigment cell receptors to circulating MSH also accelerates skin lightening, an important physiological change that aids in the prevention of overheating when the animal encounters a high ambient temperature (480). Lightening of chromatophores is supposed to be induced by the pineal-gland hormone melatonin (77), which is

interesting because melatonin is capable of lowering preferred $T_b$ in bull snakes, *Pituophis melanoleucus* (344) suggesting a common hormonal role in thermoregulation. Nonetheless, melatonin's direct role on the chromatophores is in question, since melatonin has been shown not to effect color change directly in certain reptilian species (77, 479).

Physiological color changes are also dependent on the number of melanophores present in the dermis; the array of melanophores and their relationship with iridophores and xanthophores varies in volume or distribution within the dermis in different reptilian species (344). Chromatophore type, abundance, and their capacities to activate mechanisms for physiological color changes vary among species, providing abundant evidence for natural selection (480). Nevertheless, melanophores have the principal role over their dermal counterparts (xanthophores and iridophores) and hence its presence and dominance is necessary as a means of effective thermoregulation (87, 294, 339, 408, 479).

The overall trend emerging is that basking behavior and dermal physiological responses in terrestrial ectotherms (namely, lizards and amphibians) represents a balance of trade-offs related to the regulation of body temperature, water balance, crypsis, and social interactions (170, 213). When, and if changes in reflectance can manifest and alter rates of heating and ultimately $T_b$ depends on the biotic (e.g., feeding, activity, time of year, defense behaviors, and competition over resources) and abiotic context (e.g., altitude, water availability, and ambient temperature) that the animals inhabit.

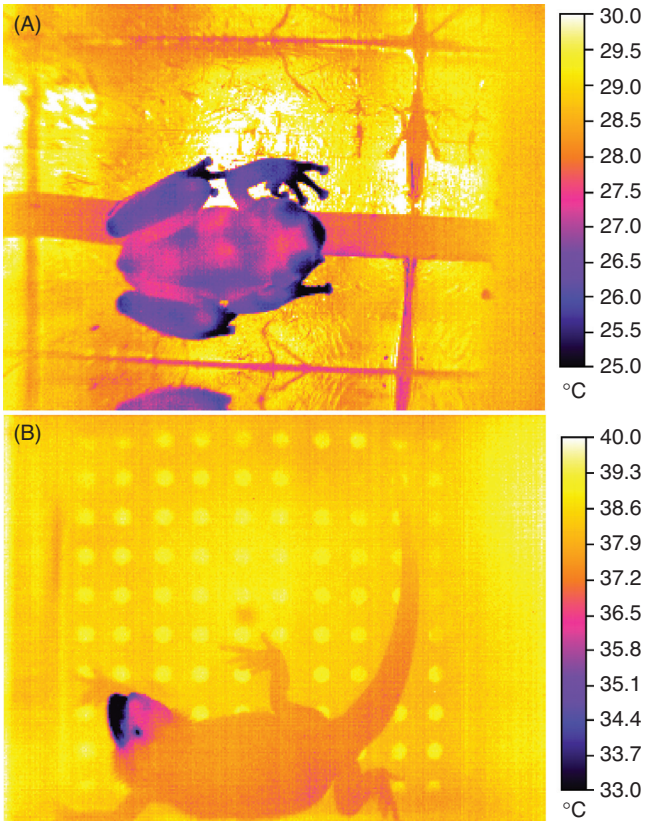


**Figure 18** Thermal images demonstrating extensive evaporative heat loss in an amphibian (A) (*Bokermannohyla alvarengai*) and evaporative cooling localized to the mouth during gaping in the bearded dragon (B) (*Pogona vitticeps*). Images courtesy of G.J. Tattersall.

### *Cutaneous evaporative heat loss*

At elevated air temperatures, when the gradient for radiative, convective, and conductive heat transfer is not favorable for the animal losing heat to the environment, the only mechanism that animals can utilize to cool down is evaporation. However, ectothermic animals do not typically possess sweat glands that would allow them to capitalize readily on cutaneous evaporative cooling mechanisms. Indeed, ectotherms can be differentiated based on their numerous strategies for avoiding evaporative cooling from the skin, rather than promoting it specifically in the manner that many mammals do. Most terrestrial insects and reptiles have relatively impermeable integuments, which minimize evaporative water loss (149, 486). Indeed, the resistance to water vapor transfer in xeric reptiles can be more than10 times higher than in nonxeric reptiles, and more than 1000 times greater than in amphibians. Amphibians have very little keratin within their skin, as well as a thin stratum corneum, in contrast to the lipid-keratin complex barriers of amniotes that exists inside the thick stratum corneum (326). Due to the high capacity for heat transfer via the latent heat of evaporation of water, the thermal physiology of animals can be highly influenced by the rate of evaporative transfer from the skin. If skin surfaces allow high water vapor movement, then heat can be exchanged with the environment in a manner rapid enough to cool the body significantly (Fig. 18A). Indeed, although many amphibians may be observed to bask in the sun, with high rates of evaporative heat transfer, body temperatures may still be several degrees Celsius below the prevailing air temperature (486).

Within the amphibians, a number of strategies exist for managing $T_b$ during elevated temperature exposure. Firstly, the rate of cutaneous evaporative heat loss generally increases exponentially with increasing air temperature, providing amphibians with a means of keeping $T_b$ lower than ambient temperature (67) but at the cost of high rates of water loss (554-556). This particular strategy cannot last without easy access to water. In a number of arboreal frogs, however, further increases in body temperature are accompanied by the secretion of lipids onto the skin's surface, which serves to minimize water vapor transfer (326). These secretions are invariably accompanied by very distinctive wiping behavior, where the animals distribute the compounds evenly across the entire skin (30, 205, 327, 328). Following this, most of these arboreal frogs adopt a typical "water-conservation posture" to minimize the rate of water loss, and remain thermally passive. In some of the so-called "waterproof" amphibians, such as *Phyllomedusa sauvagei*, cutaneous evaporative water loss rates are extremely low and nearly temperature independent, until a critical temperature is reached (487). Above this temperature, the frogs appear to "sweat," releasing glandular

secretions on the skin that act to promote evaporation and prevent $T_b$ from increasing to lethal temperatures.

Despite the general lack of sweat glands, certain reptiles, namely, desert dwelling lizards like the Gila monster and bearded dragons appear to make use of a unique mechanism of evaporative water loss. At elevated temperatures, cloacal evaporative cooling is effective at keeping $T_b$ from overheating (139). Prevention of this evaporation raises body temperature significantly, suggesting that vapor and moisture released from the cloaca is an effective means of regulating $T_b$. Hypoxic stress also appears to alter the threshold temperature for cloacal evaporative cooling in bearded dragons (539), providing further evidence for an integrated thermoregulatory control in evaporative responses.

### *Ventilatory evaporative heat loss*

At ambient temperatures above normal thermal preference many reptiles (primarily lizards and crocodilians) will pant, adopting a lower tidal volume and rapid breathing (138, 184). The mouth is typically held open and in some cases, the tongue protrudes, both of which enhance the evaporative heat loss from the moist oral and respiratory tracts (117, 137, 238). In a manner analogous to what occurs in birds and mammals, panting occurs at a critical, elevated body temperature demonstrating a clear role in thermoregulation (reviewed in reference 539). In some species (skinks, for example), mouth gaping occurs at extremely high body temperatures, and is accompanied by breathing spasms, uncoordinated body contractions, and apnea, appearing in this case to have no role in thermoregulation (570,587). Some snakes and turtles have been observed to exhibit gaping and panting at higher temperatures (270, 374), although a specific role for regulating $T_b$ has not been demonstrated. Indeed, even an amphibian has been noted to adopt panting or gaping, although to date this has only been noted in one species (328). The open mouthed gaping observed in reptiles is often accompanied by gular pumping or gular fluttering, especially in varanids and geckos, and similarly to what is observed in birds, is thought to aid in convective and evaporative heat loss from the upper airways (238, 587).

Nevertheless, gaping has been shown to be an effective thermoregulatory strategy in some reptiles. In crocodiles, for example, gaping will reduce head temperature and the rate at which brain temperature rises under heat stress, which extends basking periods required for the larger body to reach warm temperatures, while preventing the brain from overheating (511). A similar role for gaping in keeping head temperatures from overheating has been shown in several lizards (120, 144). The Chuckwalla lizard is capable of maintaining brain temperatures 3°C lower than ambient temperature for up to 8 h when it is capable of panting, whereas this effect disappears if panting is prevented (117). Whether these effects are due to physiological regulation or to the differential inertia of body parts (422), remains an untested question. Nevertheless, the close association with rapid changes in brain temperature coincident with the onset of panting strongly suggests that ventilatory evaporative water loss (Fig. 18B) is highly linked to the maintenance of brain temperature (117, 120, 376, 545).

The advantage of a ventilatory mechanism for modifying rates of heat loss is that it can be rapidly activated or terminated, depending on the body or brain temperature and environmental conditions (118, 120, 144). For example, certain internal and external influences will alter the drive to regulate body temperature, and therefore alter the threshold temperature at which gaping or panting is activated (537,539). The level of hydration can vary the threshold panting temperature (407), as well as the oxygen status (539) and sex hormones (537). Indeed, in the desert dwelling lizard, *Pogona barbata*, panting thresholds are directly related to the level of dehydration (407), allowing these lizards to precisely regulate temperature when water is abundant, while possibly abandoning such precision when water is scarce. The parallels of this system to the selective brain cooling (SBC) observed in mammals suggests a reptilian origin for the neural control of brain temperature, as well that SBC might not be only a mammalian physiological response (537).

## "Thermo-Morphological" Laws: Is there a Physiological Basis?

### Bergmann's rule

Body size has long been known to influence thermal relations in animals. For example, surface area:volume ratios, thermal inertia, metabolic capacity, and growth rates are all potentially influenced by the size of the animal (586). Changes in body size alter the conductance of body heat, and thus will influence the rate of heat exchange with the environment. This is an outcome of the physical transfer of body heat. Although altered thermal conductance can also be evoked through changes in body posture, or alterations in insulative capacity (601), Bergmann's rule was coined to accompany the observation that Bergmann made within groups (referred to initially as "races") of closely related animals demonstrating that larger individuals are observed in cold climates (high latitudes), while smaller individuals inhabit warmer habitats (44). As with any "rule" in biology, exceptions and misunderstandings have arisen, and generally, the rule should be looked at as more of a null expectation that body size will change in this manner in the absence of stronger selection pressures.

Evidence for Bergmann's rule in endotherms has most recently been observed in birds, although primarily within species (65,66). However, a prescribed survey of North American mammals, demonstrated relatively little evidence for Bergmann's rule within species (361). Subsequent studies have still found the Bergmann body size trend with latitude, but provided explanations on other energetic concerns, and made claims that body size effects are based on nonthermoregulatory basis (509); a rigorous test of the lack of a thermoregulatory role in these studies does not exist. In a more recent study examining Chukar partridges (626), it was noted that changes in body mass over the second half of the

20th century have occurred that support Bergman's rule; birds are getting smaller as the climate warms, although whether these changes in body size reflect natural selection or inherent developmental responses to temperature is unknown.

In numerous cases showing support for Bergmann's rule, selection on body size itself has been evoked as the mechanism behind the effects (65), suggesting a genetic basis. However, such a strictly adaptationist viewpoint does not consider the presence of physiological and developmental constraints that may modify energetic growth and efficiency, and influence body size indirectly (586). For example, Bergmann's rule has recently begun to be applied in certain contexts to ectothermic species (18), suggesting thermoregulation is not the only causative reason behind this effect. Indeed, Ashton and Feldman (17) claimed to be the first (although see reference 409) to demonstrate evidence for Bergmann's clines in vertebrate ectotherms (turtles); however, at the same time, they demonstrated that within the squamates, the pattern is the opposite (smaller sizes in colder climates). They suggest that this opposite trend may have a thermoregulatory basis, but is instead based on the requirement for rapid heat gain; small lizards and snakes can raise their body temperatures faster while basking, which would be of greater benefit to animals in colder environments. Angilletta et al. (8) observed Bergmann's cline in lizard species, but explain this based on life-history traits selecting for greater survival in cold environments that is bestowed to larger lizards, rather than a thermoregulatory argument. Nevertheless, the proximate (physiological) mechanism behind this response is still the delayed maturation that occurs at lower temperatures, and how that may influence selection on body size. It has been shown that changing temperature during development in many ectotherms affects the allocation and efficiency of energy stores and their use. In other words, temperature influences growth and metabolism differently, and although low temperatures lower the growth rate, it affects metabolic efficiency differentially. The net result is a larger size at maturation (564), which might be the explanation for the observed effects in ectotherms.

One of the challenges in this field has been that ecological studies rarely express their results based on proximate, developmental constraints that may contribute to the pattern, whereas physiological studies do not necessarily address the role that natural selection may play in producing the particular anatomical or physiological response. This field is also plagued by a lack of synthesis between the different physiological, morphological, developmental, behavioral, and evolutionary constraints that all influence body size and extremity size. Evoking only one of these as the putative explanation may prove to be short sighted.

## Allen's rule

Although often confused with Bergmann's rule, the influence of temperature on the development of body shape and the size of peripheral organs and appendages was first noted by Allen within species (or closely related species) inhabiting different thermal climes (2). At the time, Allen observed that extremities of (primarily) endothermic homeotherms were larger in animals from warmer climates than in animals from cold climates; in other words, strong correlations exist among latitude, ambient temperature, and limb length. Although Allen (2) did initially speculate that numerous extremities (e.g. ears, nose, limbs, and bills) would be subject to this rule, for years the primary evidence was initially anecdotal (namely fox and rabbit ears), and the emphasis for the rule was within animal races rather than as a broader adaptive response for multiple species.

Recently, data have begun to emerge for quite strong evidence for Allen's rule interspecifically, and within certain animal groups, most particularly in birds (446). Nudds and Oswald (397) examined numerous species of gulls and terns, and revealed that the size of limb elements (namely, tarsus length) were shorter in species of birds that breed in colder climates than in species that breed in warm climates. Symonds and Tattersall (531) expanded this analysis to a broader range of bird species as well as peripheral appendages, and demonstrated that when controlling for phylogeny, temperature plays a more significant role in shaping bill size than in shaping limb size (Fig. 19). Given the vastly different vascular networks that underlie limbs (countercurrent) versus bills (ramified blood supply), the authors speculate that the stronger expression of Allen's rule for bill structures may be that physiological roles for heat release or conservation may be quite different and under morphological constraints of the organs themselves.

As with Bergmann's rule, the primary explanation for Allen's rule has been genetic selection (473), where the selection on thermoregulatory adaptations has been strong. What is often overlooked is that appendage growth during

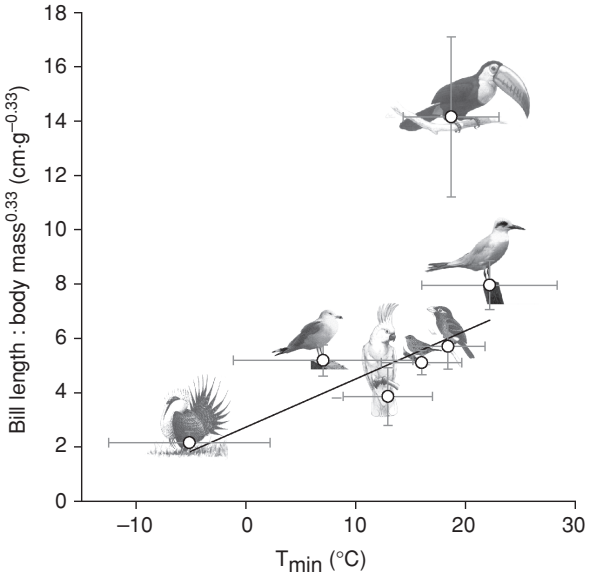


**Figure 19** Bill size in birds demonstrates a strong dependency on habitat temperature, with small bills observed in cold climates, and large bills in warmer climates (data derived, with permission, from reference 531).

development is strongly influenced by environmental temperature itself (473-475, 586). Serrat et al. (473) demonstrate that during development in rodents, the temperature of the growth plate influences chondrocyte proliferation and extracellular matrix volume. Since endotherms living at different temperatures should exhibit vasomotor changes to the peripheral tissues, this may further modulate limb and tail growth indirectly by reducing growth plate temperature. The temperatures of the internal structures are not known precisely during growth, and furthermore, the physiological control of vasomotor tone to all appendicular structures is unknown in neonatal and juvenile endotherms. For example, it has been shown that juvenile birds exhibit much higher rates of blood flow and heat loss to peripheral appendages and little vasomotor control in the cold (533), which makes a single proximate cause for the expression of the Allen's rule to be unlikely.

One challenge with both of these ecogeographical rules is a relative paucity of data regarding their mechanistic basis, and the role of physiological control. Indeed, Scholander (456) had speculated that the Allen's rule could not be explained from thermoregulatory principles in endotherms, since physiological control over peripheral blood flow would counteract any surface area effect created by differential peripheral organ size. However, this assumes that the physiological control of blood flow coevolves with the extremity size (i.e., that large appendages also have relatively greater blood supplies and exhibit lower thermal thresholds for vasodilation). Furthermore, most studies on extremity size in endotherms have focused on adult morphologies, with relatively few examining growth and within generational effects to rule out phenotypic plasticity as the primary causative agent in these rules (except see references 473 and 474). Interestingly, a recent study has also examined Allen's rule in ectotherms and found no evidence for the differential growth of protruding body parts with temperature (49). This is not surprising if Allen's rule is a thermoregulatory adaptation in homeothermic endotherms involving alteration in internal heat flow, although it is not consistent with the observation that temperature can directly influence appendage growth rates (473).

The reason why these questions, after more than 100 years of speculation, still remain important is that they are simple indicators of the aggregate role that temperature plays on animal growth, development, and physiology. Given the predicted rise in temperatures across the planet over the next 100 years, it is important to distinguish whether these morphological parameters are based on genetic adaptation, phenotypic plasticity during development, or both. In the first instance, an evolutionary mismatch may arise if appendage or body size cannot evolve quickly enough, whereas in the second instance, the environmental/physiological matching already exists.

## Conclusions

It is clear that temperature has profound effects on biochemical and cellular processes in animals. Indeed, short-term fluctuations in body temperature are unlikely to be immediately compensated by animals, requiring the input of behavioral and physiological responses to allow for the avoidance of large body temperature changes. Many ectothermic animals have evolved a range of biochemical mechanisms to cope with the wide range of body temperatures they exhibit, including the ability to express altered enzymes under different thermal environments, a reversible acclimation process to thermal environments, antifreeze proteins, ice-nucleating proteins, and numerous cryoprotectant mechanisms. Endothermic animals maintain elevated body temperatures, primarily through the use of conserved, biochemically based thermogenic mechanisms, controlled by neural mechanisms shared by most vertebrates; the fact that modern day members of the group of animals (i.e., the reptiles) thought to best resemble the ectothermic ancestors of birds and mammals have been demonstrated to possess the same physiological and behavioral thermoeffectors, supports this contention (215). Couple this with the fact that known protein markers of placental mammal endothermy (i.e., UCP1) have been found recently to exist in fish (261), suggests that the evolution of endothermic homeotherms, similar to the molecular evolution to extreme temperatures, has not involved the invention of new processes or genes, but rather the modification of existing processes and genetic material. Indeed, endothermic homeotherms like birds and mammals differ primarily in the magnitude (i.e., quantity) of the thermoregulatory responses they exhibit in response to changes in temperature, rather than the form (i.e., quality) of responses.

Given the degree of plasticity of phenotypic traits in response to temperature, what is the likelihood that body temperature and performance are coadapted? Humans and other homeotherms are extreme cases where body temperature is regulated within a very narrow range at all times and biochemical rates are also optimized within that narrow range. However, body temperatures are more variable in most other endotherms. In species that do not enter torpor, body temperatures may vary between seasons and there are concurrent shifts in the thermal sensitivity of biochemical reactions (202). Endotherms that enter torpor obviously experience pronounced fluctuations in body temperature and associated regulatory responses that vary cellular rate functions according to the stages of the torpor cycle. Indeed, perhaps the group of animals that will reveal more about the evolution of endothermic thermoregulatory mechanisms in the future are the heterothermic mammals, whether monotremes, marsupials, or the numerous mammals that have subsequently adopted heterothermy, as part of their repertoire of strategies for coping with energetic balance (100, 195).

## Perspectives

The field of thermal physiology will continue to be a dynamic and multidisciplinary discipline in the future. Driven by a current pervasive emphasis on climate change and the predicted influences of temperature on animal's abilities to respond to

environmental change, the most intriguing research will involve integrating thermal responses at multiple levels and time scales. For example, an exciting area for the future will involve the association between the neural pathways controlling $T_b$ and whether these communicate with the acclimation mechanisms that manifest within the tissues. Much work is being currently being conducted on single-celled organisms and model animals such as *C. elegans*, demonstrating "thermal memory" for acclimation history; as the neural mechanisms are uncovered in these "simple" animals, extrapolation to the more complex nervous systems of vertebrates will surely follow.

Another interesting field involves the epigenetic effects of temperature, allowing thermal histories to have a heritable component, and possibly contribute to more rapid evolutionary changes to changing environments. Ever since the arrival of the "postgenomic" era, it has become evident that the number of genes possessed by mammals underestimates the expected diversity of genetic responses, meaning that the direct encoded information is not sufficient to explain developmental, physiological, and responses involving plasticity. From a molecular regulation perspective, an untapped area for future growth is the possible role of DNA methylation and posttranscriptional regulation (e.g., alternative splicing) in thermal acclimation responses (336). Although little research has explored this particular question, splice variants may be at the heart of much of the short-term acclimatory responses that animals express when encountering changes in temperature, akin to the more specific cases of isoform thermal evolution (576).

Finally, incorporation of the concept of temperature as a natural "stressor" may change our perspectives on temperature transduction. Recent work has shown that temperature sensation mechanisms are evoked by a family of membrane bound ion channels (namely the TRP channels = transient receptor potential family of ion channels) that alter the opening probability within select ranges of temperatures (369, 589). Since this same family of ion channels also responds to pheromones, olfactory stimuli, nerve growth factor, mechanical disturbances, chemicals, pH changes, osmolarity, vasorelaxation of blood vessels, and metabolic stress, this suggests that temperature signals may be perceived similarly to a host of extracellular substances. The potential for competitive (i.e., conflictive) or even synergistic (i.e., additive) signaling between temperature and these other stimuli remains a strong possibility which will likely shape future research in the field of thermal signal transduction (325).

At present, research into how the planet's climate is undergoing rapid changes is driving much ecological, evolutionary, and physiological research. The primary goal of this research is to assess whether animals have the necessary plasticity, genetic diversity, and evolutionary capacity to respond to rapidly changing environments not only to simply survive but also to adapt to changes in the thermal environment. Since variability in the thermal physiological responses is the substrate upon which natural selection will act, the basis for physiological variability in thermal tolerance and capacity should still remain a primary concern for future research.

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