

## COMMENTARY

### Avoidance and tolerance of freezing in ectothermic vertebrates

Jon P. Costanzo\* and Richard E. Lee, Jr

Department of Zoology, Miami University, Oxford, OH 45056, USA

\*Author for correspondence (costanj@miamioh.edu)

#### Summary

**Ectothermic vertebrates have colonized regions that are seasonally or perpetually cold, and some species, particularly terrestrial hibernators, must cope with temperatures that fall substantially below 0°C. Survival of such excursions depends on either freeze avoidance through supercooling or freeze tolerance. Supercooling, a metastable state in which body fluids remain liquid below the equilibrium freezing/melting point, is promoted by physiological responses that protect against chilling injury and by anatomical and behavioral traits that limit risk of inoculative freezing by environmental ice and ice-nucleating agents. Freeze tolerance evolved from responses to fundamental stresses to permit survival of the freezing of a substantial amount of body water under thermal and temporal conditions of ecological relevance. Survival of freezing is promoted by a complex suite of molecular, biochemical and physiological responses that limit cell death from excessive shrinkage, damage to macromolecules and membranes, metabolic perturbation and oxidative stress. Although freeze avoidance and freeze tolerance generally are mutually exclusive strategies, a few species can switch between them, the mode used in a particular instance of chilling depending on prevailing physiological and environmental conditions.**

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/216/11/1961/DC1>

Key words: amphibian, hibernation, ice nucleation, inoculative freezing, reptile, supercooling.

Received 28 September 2012; Accepted 2 February 2013

#### Introduction

Ectothermic vertebrates have successfully colonized virtually every available ecological niche on Earth, some thriving in seasonally or continuously cold habitats at high latitudes and altitudes. In this commentary we briefly discuss adaptations of these animals to survive exposure to subzero temperatures and, in certain cases, the freezing of their body fluids.

Excepting certain polar fishes, which swim in ice-laden waters, most aquatic species occupy habitats that are relatively warm. Accordingly, we here focus on terrestrial species whose body temperature ( $T_b$ ) may fall appreciably below the equilibrium freezing/melting point ( $T_{Feq}$ ) of their body fluids. Some of the key tenets of cold hardiness are best illustrated from invertebrates, which are more extensively researched than higher taxa. However, we liberally reference two of the best-studied and most cold-hardy vertebrates. The wood frog (*Rana sylvatica*), the most northerly distributed of North American amphibians, occurs within the Arctic Circle. Throughout its range, this species overwinters beneath forest duff where it encounters subzero cold but nevertheless survives the freezing of its tissues. The painted turtle (*Chrysemys picta*) is a cold-adapted reptile that ranges to within 7° latitude of the Arctic Circle. Hatchlings of this species commonly overwinter within the natal nest, only 5–10 cm below the ground surface, but can survive chilling to temperatures as low as –4°C in the frozen state or –15°C by avoiding freezing.

#### Winter thermal environment

Among cold-hardy organisms, capacity for cold tolerance is tuned to the temperatures and exposure durations that a given species encounters within its habitat (Addo-Bediako et al., 2000). Thermal

conditions in winter refugia vary markedly, the intensity and frequency of chilling excursions increasing with altitude and latitude, and decreasing with any insulation afforded by the microenvironment. In temperate regions, frogs overwintering on the forest floor may encounter minima of –5 or –7°C (MacArthur and Dandy, 1982; Schmid, 1982), although more severe chilling can occur in northerly regions. Temperatures ranging between –1 and –4°C were recorded in grass tussocks harboring European common lizards, *Lacerta vivipara*, during particularly cold periods with little snow cover (Grenot et al., 2000). Where snow cover is routinely sparse and winters severe, temperatures can be, but are not necessarily, extreme. At a given study site, thermal minima within *C. picta* nests can vary by 10°C or more, reflecting differences in nest depth, slope, aspect, patchiness of snow cover, and other physiognomic factors (Costanzo et al., 2004; Weisrock and Janzen, 1999). A common but erroneous perception is that all hibernators must endure a single, winter-long bout of extreme cold. The more accurate scenario, especially for animals using well-insulated refugia, is for the temperature to hover near 0°C for extended periods that may (or may not) be punctuated by brief (i.e. hours to days) excursions to slightly lower temperatures before rewarming (Costanzo et al., 2008; Grenot et al., 2000). Nevertheless, where snow cover is scanty and winters are severe, frost may descend to a considerable depth and persist for extended periods.

#### Strategies for coping with extreme cold

Species that cannot evade frost depend on either of two survival mechanisms: freeze avoidance through supercooling or freeze tolerance. With few exceptions, these are mutually exclusive

strategies. Some authors posit various phylogenetic, ontogenetic and ecological arguments that freeze avoidance is the basal modality, with freeze tolerance being a recently evolved trait (Sinclair et al., 2003). Both strategies present unique stresses and limitations. For example, whereas freeze tolerance allows revival after the tissues have frozen and thawed, it offers protection at relatively modest temperatures. By contrast, a strategy of supercooling permits survival over a broader temperature range but is inherently precarious: freezing – with ensuing death – can occur spontaneously at any temperature below the  $T_{Feq}$  (Fig. 1). Another freeze-avoidance tactic, cryoprotective dehydration, eliminates freezing risk but is effectual only in small ectotherms possessing a highly permeable integument and profound desiccation tolerance (Holmstrup et al., 2002). Conceivably it could be used by certain amphibians, but is as yet unknown among vertebrates.

### Regulation of ice nucleation

Control of ice nucleation is critical regardless of whether an organism employs a strategy of freeze avoidance or freeze tolerance. Regulation is achieved by managing the abundance and/or potency of ice-nucleating agents (INA) in or on the body, and by modulating the propensity for inoculative freezing occurring through intimacy with ice and INA in the environment (Costanzo and Lee, 1995; Costanzo and Lee, 1996; Lee and Costanzo, 1998).

Freeze-avoiding species commonly prepare for winter by eliminating alimentary matter that could initiate freezing within the gut (Lee, 2010). For example, hatchling *C. picta* harbor INA that, derived from internalized yolk, are eliminated with feces following hatching (Fig. 2A). Freeze-avoiding species must evade a host of INA that naturally occur in overwintering environments, as these mineral particulates (e.g. quartz and silicates), organic crystalloids and organic/inorganic complexes formed during decay, and certain bacteria and fungi may access body fluids through orifices or wounds, thereby triggering ice nucleation (Vali, 1995). Even brief physical contact with such agents can reduce supercooling capacity by up to 10°C in hatchling turtles (Fig. 2B).

Terrestrial hibernators are often subject to contact with ambient ice, which can initiate the freezing of their body fluids. The risk of inoculative freezing increases as  $T_b$  falls and with the passage of time, but is also modulated by environmental factors that influence an organism's intimacy with ice and the propensity for ice to access tissues (Fig. 3). Amphibians are particularly susceptible to inoculative freezing because ice readily permeates their moist skin (Layne, 1991). The integument of fishes and reptiles better resists ice transmission, but is an imperfect barrier. Even if direct contact with ice can be avoided, freezing could be triggered by ice crystals growing in a plume of water vapor issued from pores in the body surface (Salt, 1963).

Freeze-tolerant species commonly initiate freezing at a  $T_b$  nearest the tissue  $T_{Feq}$  to avoid damage from rapid, uncontrolled ice

growth (Mazur, 2004; Storey and Storey, 1988). Towards that end, some synthesize inorganic crystals or, more commonly, ice-nucleating proteins (INP) that organize water molecules through hydrogen bonding to form an ice embryo. Although the blood of freeze-tolerant vertebrates lacks biologically efficient INP (Costanzo and Lee, 1996), it remains untested whether such agents are associated with cellular structures, such as the plasma membrane (see Izumi et al., 2006). However, in these organisms control of ice nucleation is primarily attained through inoculative freezing by contact with external ice or INA. Some also use auxiliary mechanisms; for example, the gut of overwintering *R. sylvatica* harbors ice-nucleating bacteria, including *Pseudomonas fluorescens*, *P. putida* and *Enterobacter agglomerans*, which can initiate tissue freezing near –3°C (Lee and Costanzo, 1998). In addition, skin secretions of the brown tree frog (*Litoria ewingii*) express ice-nucleating activity that increases in winter (Rexer-Huber et al., 2011).

### Supercooling as a survival strategy

A solution does not necessarily freeze at its  $T_{Feq}$ , but potentially can remain liquid when cooled well below this mark. Indeed, a small volume of pure water can be supercooled to nearly –40°C before spontaneously freezing (Vali, 1995). Accordingly, many ectotherms, particularly individuals weighing <60 g, can supercool appreciably (Costanzo and Lee, 1995). However, supercooling capacity is limited ( $T_c \geq -3^\circ\text{C}$ ) in woodland frogs, largely owing to their moist skin (Layne, 1991; MacArthur and Dandy, 1982). It is somewhat greater (to approximately –6°C) in the spadefoot toad, *Scaphiopus bombifrons*, which potentially avoids freezing by burrowing in relatively dry soil (Swanson and Graves, 1995). Among vertebrates, supercooling capacity is best developed in small reptiles, such as lizards and hatchling turtles, which, depending upon species, can reach –8 to –18°C before they spontaneously freeze. Within this group, terrestrial hibernators supercool more extensively than either aquatic hibernators or species that hibernate below the frost's decent (Costanzo et al., 2008). Inexplicably, the exceptional supercooling capacity of hatchling *C. picta* – to –20°C in some individuals – greatly exceeds that of similarly sized ectotherms and is on par with that of a water droplet.

Freeze-avoiding species commonly enhance their supercooling capacity by accumulating compatible osmolytes, including polyols, sugars and certain amino acids, which colligatively depress tissue  $T_{Feq}$  (Lee, 2010; Storey and Storey, 1988). Cold hardening sometimes reflects a seasonal dehydration, which serves to reduce water volume and concentrate extant and newly mobilized osmolytes in tissues. During winter acclimatization, for example, hatchling *C. picta* increase their plasma osmolality by 60–70 mosmol l<sup>-1</sup>, the rise reflecting a twofold to threefold increase in urea. This response, coupled with a reduction in body water, has a profound effect on supercooling capacity (Costanzo et al., 2004).

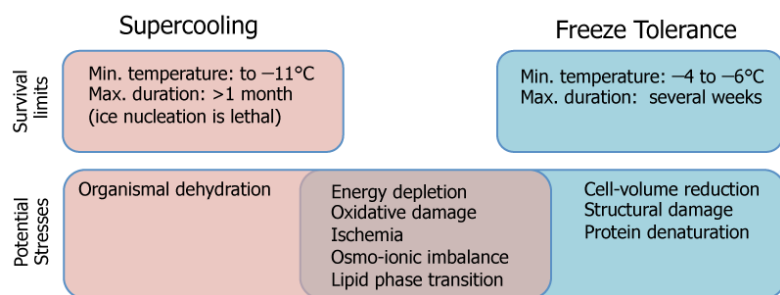


Fig. 1. Characteristics of two cold-hardiness mechanisms, freeze avoidance through supercooling and freeze tolerance, used by vertebrate ectotherms.

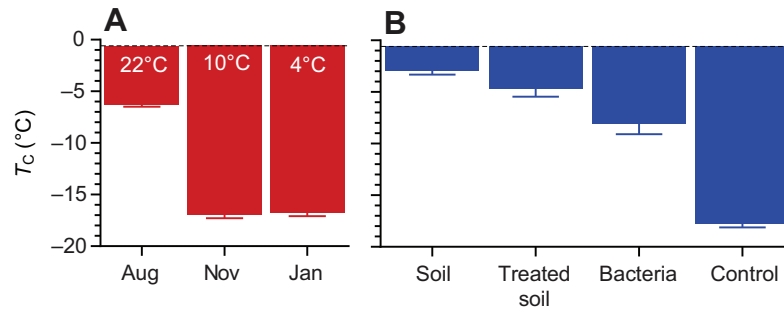


Fig. 2. Role of endogenous and exogenous INA in regulating ice nucleation in hatchling painted turtles, *Chrysemys picta*. Supercooling capacity is represented by the temperature of crystallization ( $T_c$ ) of individuals cooled in a dry, ice-nucleating agent (INA)-free environment. Horizontal dashed line represents the equilibrium freezing/melting point ( $T_{\text{Feq}}$ ) of body fluids,  $-0.6^\circ\text{C}$ , below which supercooling can occur. (A) Supercooling capacity of turtles tested in August, a few weeks after hatching, in late November, and in late January. Prevailing acclimation temperature is shown. Means  $\pm$  s.e.m.;  $N=6$ . Adapted from Costanzo et al. (Costanzo et al., 2000b). (B) Variation in supercooling capacity of winter-acclimatized turtles experimentally exposed to nesting soil containing both inorganic and organic INA; nesting soil that was autoclaved to inactivate organic INA; medium containing a killed, lyophilized preparation of the ice-nucleating bacterium *Pseudomonas syringae*; or INA-free medium (control). Means  $\pm$  s.e.m.;  $N=5-14$ . Adapted from Costanzo et al. (Costanzo et al., 2000a).

Whereas a cold-hardiness strategy predicated on supercooling has its advantages, it also carries serious risks. Most importantly, because a supercooled solution is metastable, spontaneous ice nucleation can occur at any temperature below the solution's  $T_{\text{Feq}}$ , the likelihood of this outcome increasing over time and with deeper cooling (Vali, 1995). Furthermore, extended or extreme cooling can induce injury, even if tissues never freeze. Indeed, reptiles supercooled to  $\leq -10^\circ\text{C}$  recover slowly, if at all (Costanzo et al., 2008). Causes of such chilling injury include disturbance of ion homeostasis and metabolic functions, adverse lipid phase changes in membranes, and oxidative stress (Lee, 2010; Macmillan and Sinclair, 2011). Diminished tissue perfusion at low  $T_b$  can lead to lactate accumulation, reduced intracellular pH and generation of reactive-oxygen species (ROS), although cold-hardy reptiles apparently can avoid oxidative stress (Hermes-Lima and Zenteno-Savín, 2002). Chilling injury in hatchling *C. picta* apparently does not reflect widespread membrane

damage, but seems restricted to particularly sensitive cells, such as neurons. Muir et al. (Muir et al., 2010a) examined brain tissue from cold-shocked turtles (i.e. held supercooled for 24 h at  $-13^\circ\text{C}$ ), finding that the cells had sustained direct chilling injury. In a separate experiment, preconditioning turtles at an intermediate temperature ( $-7^\circ\text{C}$ ) markedly improved their tolerance to the cold shock, a result reminiscent of the rapid cold-hardening phenomenon of various arthropods that potentially derives from membrane remodeling (Lee, 2010).

### Freeze tolerance as a survival strategy

Freeze tolerance is a multi-faceted, complex trait that has evolved independently many times in diverse organisms. Long known in arthropods and other invertebrates, it was first reported in vertebrates only some 30 years ago (Schmid, 1982). It is currently known in anuran and urodele amphibians, both suborders of

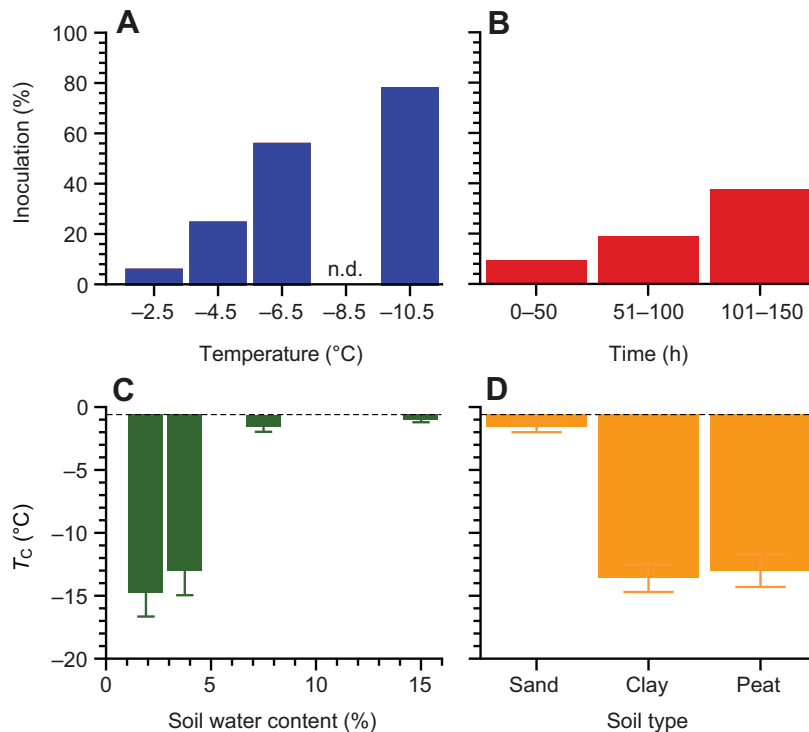


Fig. 3. Influence of extrinsic variables on risk of inoculative freezing in hatchling painted turtles, *Chrysemys picta*. (A) Inoculation frequency varied with the temperature at which turtles were held supercooled over 11 days ( $N=32$  turtles per group; n.d., not determined). (B) Inoculation frequency increased over time as turtles were held supercooled at  $-6.5^\circ\text{C}$ ; additional turtles (19%,  $N=32$ ) were inoculated before attaining  $-6.5^\circ\text{C}$ . In A and B, turtles were cooled inside artificial nests in intimate contact with frozen nest soil (loamy sand). Adapted from Packard et al. (Packard et al., 1997). Influence of soil moisture (C) and soil texture at 7.5% moisture content (D) on propensity for inoculative freezing, as gauged by the temperature of crystallization ( $T_c$ ) of individuals ( $N=3-5$  per group) cooled in a dry, ice-nucleating agent (INA)-free environment. Horizontal dashed line represents the equilibrium freezing/melting point ( $T_{\text{Feq}}$ ) of body fluids,  $-0.6^\circ\text{C}$ , below which supercooling can occur. Adapted from Costanzo et al. (Costanzo et al., 1998).

Squamata, and neonatal and adult turtles. Phylogenetic, geographic and ecological associations in vertebrate freeze tolerance are promising areas for future research.

The term freeze tolerance denotes an ability to survive the freezing and thawing of a biologically significant quantity of body water under thermal and temporal conditions of relevance to an organism's life history. Unfortunately, the literature is burdened with reports that fail to distinguish recovery from superficial freezing from a response that is truly adaptive in the sense of evolutionary import. Freeze tolerance is usually considered a strategy for surviving winter, although it may also benefit some species, such as the eastern box turtle (*Terrapene carolina*) and common garter snake (*Thamnophis sirtalis*), by safely extending their activity into the cooler months.

### Tolerance limits

Freeze-tolerant animals only survive the freezing of water within the vascular and extracellular spaces, as with few exceptions intracellular freezing is lethal. They can tolerate as much as two-thirds of their total body water being frozen, although this limit is reached at markedly different temperatures in diverse taxa (Storey and Storey, 1988). Generally, freeze tolerance is more robust in winter than during other seasons, commonly with less ice forming and less injury manifested. As a rule, minimum tolerable temperatures are more modest in vertebrates than in invertebrates, the latter extending to below  $-80^{\circ}\text{C}$  (Lee, 2010). Most woodland frogs survive experimental freezing at minima of  $-2$  to  $-6^{\circ}\text{C}$  depending upon taxon, population, age and acclimatization state, and the critical minima for reptiles (particularly squamates) are even more modest, reaching only approximately  $-4^{\circ}\text{C}$  for certain hatchling and adult turtles. Nevertheless, preliminary findings (Middle and Barnes, 2001) and ongoing work in our laboratory indicate that *R. sylvatica* indigenous to Interior Alaska, near the species' northern limit of distribution, can tolerate freezing to  $-16^{\circ}\text{C}$  or below. Moreover, exceptional tolerance, to  $-35^{\circ}\text{C}$ , has been reported for two salamanders (*Salmandrella keyserlingii* and *S. schrenckii*) indigenous to Asian Russia (Berman et al., 1984; Berman et al., 2010).

Freeze-tolerance capacity is also determined by survivable duration, which reflects the ability to cope with additional stresses, such as prolonged hypoxia and energy depletion. Limits of freeze endurance in vertebrates are poorly delineated. Frogs can withstand sustained freezing for perhaps many weeks, depending on  $T_b$  and cryoprotectant level (Layne et al., 1998). Freeze endurance in reptiles is comparatively poor, although hatchling *C. picta* can survive experimental freezing at  $-2.5^{\circ}\text{C}$  for at least 11 days and, potentially, for more than 1 month (Costanzo et al., 2008).

### Biophysical and physiological responses

Below we highlight some salient aspects of the freezing and thawing dynamic. The transformation is perhaps best appreciated by directly observing a frog or turtle succumb to freezing and reanimate after thawing, and we invite the reader to view a time-lapse video of the thawing sequence (see supplementary material Movie 1).

Ice nucleation and the ensuing period of freezing generate heat that suspends cooling of the specimen and induces a thermal plateau in which  $T_b$  approaches the  $T_{\text{Feq}}$  (Fig. 4). During this exothermic phase, which may last from hours to days, cardiovascular function is maintained and cells mount protective defenses as ice pervades the peripheral tissues (Layne et al., 1989; Storey and Storey, 1988). With continued cooling, freezing progresses and  $T_b$  falls to the limit allowed by the environment. At this stage, ice amasses within the coelomic and lymphatic spaces; heart rate declines and circulation is lost to all but the core organs. Ultimately, muscular, pulmonary and cardiac activities cease.

Thawing essentially reverses the freezing dynamic, with melting beginning in the core organs (Rubinsky et al., 1994). Cardiac contractions resume even before thawing is complete; yet how the heart performs under hypoxia and low venous return remains unknown. Pulmonary ventilation and tissue perfusion then return, followed by nerve excitability and motor faculties. Simple reflexes are restored before more complex neurobehavioral functions, such as locomotion and reproductive behaviors (Costanzo et al., 1997b). Most faculties are restored within a day or two of thawing, although a longer recuperation is needed following more severe freezing (Layne et al., 1998).

### Freezing injury and adaptive responses

The magnitude of freezing/thawing stress is governed by exposure temperature (and associated ice content), exposure duration and cooling rate; yet survival of even a mild freezing episode demands tolerance of multiple stresses simultaneously manifested at all levels of biological organization. Perturbations stem from osmotic withdrawal of cytoplasmic water, leading to excessive cell shrinkage and untoward hyperosmotic/ionic effects on macromolecules, membranes and cell homeostasis (Mazur, 2004; Storey and Storey, 1988). Freezing alters the physical state of lipids and hampers membrane-mediated processes, such as solute transport, iono-osmotic regulation and intercellular communication. Impairment of cellular gas exchange leads to hypoxia and oxidative damage, and pervasion of ice within tissues disrupts their architecture, damaging capillaries and triggering ischemic necrosis or apoptosis (Hoffman and Bischof, 2004).

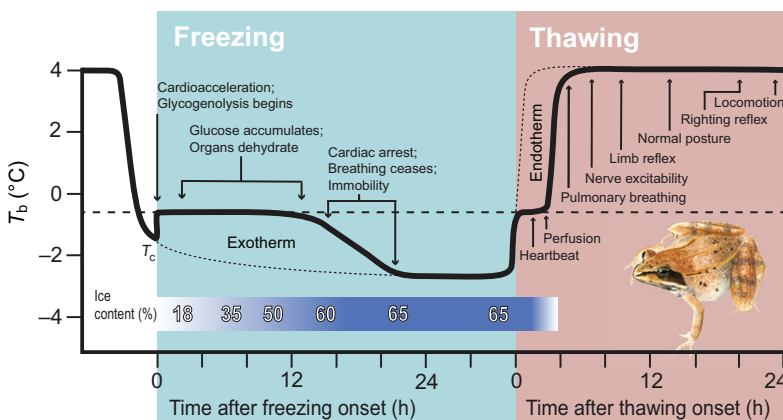


Fig. 4. Typical freezing and thawing time course during an episode of cooling from  $4$  to  $-2.5^{\circ}\text{C}$  and subsequent return to  $4^{\circ}\text{C}$  in the wood frog, *Rana sylvatica*, illustrating key physiological responses in relation to dynamics of ice within the body. Freezing begins with the ice nucleation event after supercooling to approximately  $-2^{\circ}\text{C}$ .

Prolonged freezing exacerbates stress by depleting metabolic fuels, accumulating wastes, such as lactate, and promoting recrystallization (Costanzo et al., 2008; Knight and Duman, 1986; Layne et al., 1998).

Freeze-tolerant organisms invoke a suite of coordinated molecular and physiological responses to minimize injury caused directly or indirectly by the formation and melting of ice (Storey and Storey, 1988). Exapted from fundamental responses to osmotic, hydric and hypoxic stresses, these include mechanisms to manage the distribution of water and solutes, conserve energy and mediate metabolism, enhance antioxidant defenses, and upregulate genes involved in preserving homeostasis and somatic repair. Below, we highlight some of the key adaptive responses.

### Gene and protein responses

Innovative techniques in molecular biology, including construction and screening of cDNA libraries, microarray screening and reverse transcription polymerase chain reaction (RT-PCR), have leveraged efforts to discover the gene and protein responses supporting natural freeze tolerance. Freezing alters expression of various genes, including ones involved in cell repair, metabolic suppression and antioxidation, as well as ones encoding novel proteins (Storey, 2004). Much of this research has focused on *R. sylvatica*, although hatchling *C. picta* also exhibit freezing-responsive genes, the products of which include antioxidant enzymes, inhibitors of serine protease and iron-binding proteins (Storey, 2006). An important theme emanating from this research is that various genes activated by anoxia or desiccation also respond to freeze/thaw stress, bolstering the view that freeze tolerance was derived from fundamental mechanisms to mitigate cell-volume reduction and cope with lack of oxygen.

### Cryoprotective solutes

Organic osmolytes accumulated before, during or perhaps after freezing play important roles in protecting cells and tissues from freeze/thaw stresses (Lee, 2010; Storey and Storey, 1988). Representing several classes of compounds, these solutes are of low molecular mass and are benign in high concentrations. Permeating solutes, including polyhydric alcohols and some sugars, not only colligatively reduce ice formation, but also limit cell shrinkage as tissues freeze. Some cryoprotectants, including certain amino acids, function at relatively low concentrations to fortify membranes and macromolecules. Still others serve as antioxidants or metabolic substrates or regulators (Table 1).

Many freeze-tolerant organisms accumulate cryoprotectants in anticipation of freezing, typically during seasonal cold hardening (Lee, 2010; Storey and Storey, 1988). Among vertebrate ectotherms, this response is exemplified by members of the gray treefrog complex, which, upon cold acclimation, can raise

plasma glycerol concentration from  $\sim 1$  to 50–80 mmol l<sup>-1</sup> or more (Layne and Stapleton, 2009; Zimmerman et al., 2007). Similarly, glycemic levels in winter exceed those in autumn by some 15-fold (to 60 mmol l<sup>-1</sup>) in hatchling *C. picta* (Costanzo et al., 2008) and at least fourfold (to 50 mmol l<sup>-1</sup>) in *L. vivipara* (Grenot et al., 2000). Woodland frogs can accumulate urea in tissues (potentially >90 mmol l<sup>-1</sup> plasma) in autumn and early winter owing to hydric stress, cold-induced oliguria and high activity of the regulatory enzyme carbamoyl phosphate synthetase I (Schiller et al., 2008). Urea also accumulates (to 30–80 mmol l<sup>-1</sup> plasma) during cold acclimation in various species of hatchling turtles (Costanzo et al., 2008). Besides its cryoprotective role, urea, by virtue of its destabilizing effects on proteins, may contribute to the hypometabolic state of hibernating ectotherms. According to this idea, transition between arousal and dormancy is modulated by shifts between the active/inactive states of key regulatory enzymes in response to changing urea concentration, pH and temperature. In *R. sylvatica*, elevated urea is associated with depressed metabolism in isolated tissues and intact animals (Muir et al., 2008); this system of metabolic control, which may be prevalent among ureogenic ectotherms that do not co-accumulate methylamines or other counteracting solutes (Muir et al., 2010b), is an exciting area for future research.

Seminal studies of freeze tolerance in woodland frogs have documented their efficacious system for copiously mobilizing glucose or glycerol in direct response to corporal freezing (Schmid, 1982; Storey and Storey, 1988). Cryoprotectant is chiefly derived from glycogen that accumulates in liver, representing up to 20% of organ mass, during late summer and autumn. Triggered by ice nucleation, its synthesis and export to other tissues proceeds rapidly, with concentrations in blood and core organs ultimately reaching 100–250 mmol l<sup>-1</sup>. Such levels are readily tolerated by these frogs, but would be deleterious in endotherms. Upon thawing, cryoprotectant is returned to the liver and reconverted to glycogen; reabsorption of glucose in the urinary bladder assists this process and limits the excretory loss of this valuable solute (Costanzo et al., 1997a).

Biochemical control of glycogenolysis in freezing has been thoroughly investigated, particularly in *R. sylvatica* (Storey and Storey, 1988). Key features of the process include activation of glycogen phosphorylase through cAMP stimulation of protein kinase, and an inhibitory block on glycolysis at phosphofructokinase that maximizes glucose output. The glucogenic response is virtually immediate, as the liver mobilizes cryoprotectant within minutes of freezing onset. Freeze-tolerant reptiles also synthesize osmolytes (chiefly glucose and lactate) with freezing, although the levels achieved in frozen tissues, typically 15–40 mmol l<sup>-1</sup>, are far lower than those found in woodland frogs (Costanzo et al., 2008; Storey, 2006).

The importance of organic osmolytes in freezing survival has been amply demonstrated by administering putative cryoprotectants to intact animals or media containing isolated cells or tissues in advance of experimental freezing. In *R. sylvatica*, for example, physiological concentrations (40–80 mmol l<sup>-1</sup>) of urea reduce *in vitro* cryoinjury to erythrocytes and organs, enhance contractile performance of isolated gastrocnemius following experimental freezing/thawing, and improve freezing survival and post-thaw recovery (Costanzo and Lee, 2008). Generally, cryoprotectants cannot confer freeze tolerance to innately freeze-intolerant species; however, one exception to this rule is *Drosophila melanogaster*, a weakly cold-tolerant insect of tropical origin

Table 1. Some characteristics of cryoprotectants used by freeze-tolerant vertebrates

	Glycerol	Glucose	Lactate	Urea	Amino acids
Pre-freeze accumulation	✓			✓	✓
Post-freeze accumulation	✓	✓	✓		
Colligative effects	✓	✓	✓	✓	
Membrane stabilization	✓	?		?	✓
Metabolic fuel	✓	✓			
Metabolic regulation				✓	

✓ denotes the agent has the indicated trait; ? denotes the association is presumed.

whose larvae can be made to survive freezing at  $-5^{\circ}\text{C}$  simply by augmenting their diet with the free amino acid proline (Košťál et al., 2012).

#### Dynamics and distribution of water

In preparing for winter some species undergo a partial dehydration that serves to concentrate osmolytes in tissues, thereby reducing tissue  $T_{\text{Feq}}$  and the amount of ice that can form at any given  $T_{\text{b}}$ . Some also increase the proportion of ‘bound water’, water that is, by virtue of its close association with macromolecules and cellular structures, virtually unfreezeable at temperatures of physiological relevance (Lee and Costanzo, 1998). Besides these preparations, freezing induces a protective response in which bulk water translocates from tissues to the coelom and subdermal spaces where it innocuously accumulates as ice. This beneficial extra-organ sequestration of ice removes much of the water within tissues (e.g. 20–35% in appendicular muscles; >50% in core organs), causing organs to visibly shrink (Rubinsky et al., 1994). Rehydration occurs within hours after thawing commences.

#### Recrystallization inhibition

First identified in tissues of polar teleosts, antifreeze proteins (AFP) are best known for their role in preventing freezing by adsorbing to the growth front of ice embryos (DeVries, 1982). However, they also inhibit recrystallization, a thermodynamic phenomenon in which some ice crystals gradually enlarge at the expense of others, ultimately damaging tissue architecture and disrupting intercellular communication systems (Knight and Duman, 1986). Whether AFP serve this function in vertebrate ectotherms is unknown, although their blood lacks thermal hysteresis (Costanzo and Lee, 1996). Recently, a new class of antifreeze, a xylomannan-based glycolipid, was described from various freeze-tolerant organisms, including the frog *R. lessonae* (Walters et al., 2011). In this species, recrystallization inhibition is expressed in muscle and skin, but not in blood plasma, suggesting that this agent protects cell membranes from thermotropic damage and/or prevents ice from infiltrating the cytosol.

#### Ischemia, anoxia and reoxidation

Freeze-tolerant vertebrates exhibit a well-developed antioxidant defense system that minimizes oxidative damage with freezing/thawing (Hermes-Lima and Zenteno-Savín, 2002). This is important because ice accumulating in tissues causes circulation to fail, rendering cells absent of oxygen, high in lactate, and acidotic; reperfusion of tissues upon thawing promulgates oxidative stress, which can develop if production of ROS in the mitochondrial respiratory chain overtaxes antioxidant defense mechanisms. Left unchecked, ROS can oxidize macromolecules or initiate lipid peroxidation that damages cell membranes. Species prone to oxidative stress commonly maintain high levels of antioxidants, although some also mobilize defenses in anticipation of, or in direct response to, increased ROS production. Towards that end, *R. sylvatica* expresses more peroxiredoxin, an enzyme that chemically reduces organic hydroperoxides and hydrogen peroxide, in winter than in summer (Kiss et al., 2011).

#### Freeze avoidance or freeze tolerance?

Freeze avoidance and freeze tolerance are both effective cold-hardiness strategies, yet it remains unclear why a particular taxon adopts one over the other. One key determinant is the nature of the organism’s thermal environment; for example, freeze tolerance is particularly advantageous in habitats where winter temperatures are

relatively mild and unpredictable (Sinclair et al., 2003). Physical characteristics of the hibernaculum influencing freezing risk are also important, as are certain life-history traits, such as body size. Physiological constraints, including need to conserve water and energy, and limit the potential for significant cryoinjury, are also important (Block, 1991; Voituron et al., 2002).

Although freeze avoidance and freeze tolerance are generally mutually exclusive strategies, a few species present an interesting exception in that, depending upon prevailing physiological/environmental conditions, they can utilize either modality. Among vertebrates, these include hatchling *C. picta* and *L. vivipara*. This bimodal strategy, which affords plasticity in response to environmental variability, has probably allowed both species to extend their broad geographical ranges. Intensive study of these species may provide new insights into how the various selective pressures that shape life-history traits drive the evolutionary development of cold-hardiness strategies.

#### Glossary

##### Chilling injury

Injury induced by cold exposure in the absence of freezing.

##### Colligative

Depending on the quantity, but not the nature, of particles in a solution.

##### Cryoprotectant

Solute that improves cold tolerance through colligative and/or noncolligative properties.

##### Equilibrium freezing/melting point ( $T_{\text{Feq}}$ )

Maximum temperature at which a solution can freeze upon cooling and melt upon warming.

##### Freeze tolerance

Evolved trait permitting an organism to survive the freezing and thawing of a biologically significant quantity of its body water under ecologically relevant thermal and temporal conditions.

##### Hydric stress

Perturbation caused by water deficiency.

##### Ice-nucleating agent (INA)

Any naturally occurring substance, including organic and inorganic entities, that can initiate the freezing of a solution by virtue of its ability to orient water molecules to form an ice embryo.

##### Ice-nucleating protein (INP)

Specially adapted protein or glycoprotein expressing ice-nucleating activity.

##### Inoculative freezing

Process in which a solution is induced to freezing through physical contact with ice or INA.

##### Oliguria

Diminished urine formation.

##### Reactive oxygen species (ROS)

Chemically reactive, oxygen-containing compounds, such as hydrogen peroxide and various free radicals, which can cause oxidative stress and damage cellular structures.

##### Recrystallization

Time- and temperature-dependent process by which some ice crystals enlarge at the expense of smaller ones.

##### Supercooling

Metastable state in which a solution has cooled below its  $T_{\text{Feq}}$  without undergoing a phase transition. Supercooling capacity reflects the range in temperature between  $T_{\text{Feq}}$  and  $T_{\text{c}}$ .

##### Temperature of crystallization ( $T_{\text{c}}$ )

Temperature at which ice nucleation occurs in a solution at or below its  $T_{\text{Feq}}$ .

#### Acknowledgements

We dedicate this paper to William D. Schmid for his pioneering work on the winter physiological ecology and discovery of freeze tolerance in vertebrate ectotherms.

We thank Yuta Kawarasaki for offering constructive comments on the manuscript, and Michael Wright for assisting with construction of the graphics.

### Author contributions

J.P.C. and R.E.L. conceived the project. J.P.C. researched the literature, drew the figures, and drafted the manuscript with advice from R.E.L. Both authors edited the text.

### Competing interests

No competing interests declared.

### Funding

We gratefully acknowledge support for our research by the US National Science Foundation.

### References

- Addo-Bediako, A., Chown, S. L. and Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proc. Biol. Sci.* **267**, 739-745.
- Berman, D. I., Leirikh, A. N. and Mikhailova, E. I. (1984). Winter hibernation of the Siberian salamander *Hynobius keyserlingi*. *J. Evol. Biochem. Physiol.* **20**, 323-327.
- Berman, D. I., Leirikh, A. N. and Meshcheryakova, E. N. (2010). The Schrenck newt (*Salamandrella schrenckii*, Amphibia, Caudata, Hynobiidae) is the second amphibian that withstands extremely low temperatures. *Dokl. Biol. Sci.* **431**, 131-134.
- Block, W. (1991). To freeze or not to freeze? Invertebrate survival of sub-zero temperatures. *Funct. Ecol.* **5**, 284-290.
- Costanzo, J. P. and Lee, R. E. (1995). Supercooling and ice nucleation in vertebrates. In *Biological Ice Nucleation and Its Applications* (ed. R. E. Lee, G. J. Warren and L. V. Gusta), pp. 221-237. St Paul, MN: American Phytopathological Society Press.
- Costanzo, J. P. and Lee, R. E. (1996). Mini review: ice nucleation in freeze-tolerant vertebrates. *Cryo Lett.* **17**, 111-118.
- Costanzo, J. P. and Lee, R. E., Jr (2008). Urea loading enhances freezing survival and postfreeze recovery in a terrestrially hibernating frog. *J. Exp. Biol.* **211**, 2969-2975.
- Costanzo, J. P., Callahan, P. A., Lee, R. E., Jr and Wright, M. F. (1997a). Frogs reabsorb glucose from urinary bladder. *Nature* **389**, 343-344.
- Costanzo, J. P., Irwin, J. T. and Lee, R. E., Jr (1997b). Freezing impairment of male reproductive behaviors of the freeze-tolerant wood frog, *Rana sylvatica*. *Physiol. Zool.* **70**, 158-166.
- Costanzo, J. P., Litzgus, J. D., Iverson, J. B. and Lee, R. E., Jr (1998). Soil hydric characteristics and environmental ice nuclei influence supercooling capacity of hatchling painted turtles *Chrysemys picta*. *J. Exp. Biol.* **201**, 3105-3112.
- Costanzo, J. P., Litzgus, J. D., Iverson, J. B. and Lee, R. E. (2000a). Ice nuclei in soil compromise cold hardiness of hatchling painted turtles, *Chrysemys picta*. *Ecology* **81**, 346-360.
- Costanzo, J. P., Litzgus, J. D., Iverson, J. B. and Lee, R. E., Jr (2000b). Seasonal changes in physiology and development of cold hardiness in the hatchling painted turtle *Chrysemys picta*. *J. Exp. Biol.* **203**, 3459-3470.
- Costanzo, J. P., Dinkelacker, S. A., Iverson, J. B. and Lee, R. E., Jr (2004). Physiological ecology of overwintering in the hatchling painted turtle: multiple-scale variation in response to environmental stress. *Physiol. Biochem. Zool.* **77**, 74-99.
- Costanzo, J. P., Lee, R. E., Jr and Uitsch, G. R. (2008). Physiological ecology of overwintering in hatchling turtles. *J. Exp. Zool.* **309**, 297-379.
- DeVries, A. L. (1982). Biological antifreezes in coldwater fishes. *Comp. Biochem. Physiol.* **73A**, 627-640.
- Grenot, C. J., Garcin, L., Dao, J., Hérold, J., Fahys, B. and Tséré-Pagès, H. (2000). How does the European common lizard, *Lacerta vivipara*, survive the cold of winter? *Comp. Biochem. Physiol.* **127A**, 71-80.
- Hermes-Lima, M. and Zenteno-Savín, T. (2002). Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp. Biochem. Physiol.* **133C**, 537-556.
- Hoffman, N. E. and Bischof, J. C. (2004). Mechanisms of injury caused by *in vivo* freezing. In *Life in the Frozen State* (ed. B. Fuller, N. Lane and E. E. Benson), pp. 455-481. Boca Raton, FL: CRC Press.
- Holmstrup, M., Bayley, M. and Ramlöv, H. (2002). Supercool or dehydrate? An experimental analysis of overwintering strategies in small permeable arctic invertebrates. *Proc. Natl. Acad. Sci. USA* **99**, 5716-5720.
- Izumi, Y., Sonoda, S., Yoshida, H., Danks, H. V. and Tsumuki, H. (2006). Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *J. Insect Physiol.* **52**, 215-220.
- Kiss, A. J., Muir, T. J., Lee, R. E., Jr and Costanzo, J. P. (2011). Seasonal variation in the hepatoproteome of the dehydration and freeze-tolerant wood frog, *Rana sylvatica*. *Int. J. Mol. Sci.* **12**, 8406-8414.
- Knight, C. A. and Duman, J. G. (1986). Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role. *Cryobiology* **23**, 256-262.
- Košťál, V., Šimek, P., Zahradníčková, H., Cimlová, J. and Štětina, T. (2012). Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *Proc. Natl. Acad. Sci. USA* **109**, 3270-3274.
- Layne, J. R. (1991). External ice triggers freezing in freeze-tolerant frogs at temperatures above their supercooling point. *J. Herpetol.* **25**, 129-130.
- Layne, J. R., Jr and Stapleton, M. G. (2009). Annual variation in glycerol mobilization and effect of freeze rigor on post-thaw locomotion in the freeze-tolerant frog *Hyla versicolor*. *J. Comp. Physiol. B* **179**, 215-221.
- Layne, J. R., Jr, Lee, R. E., Jr and Heil, T. L. (1989). Freezing-induced changes in the heart rate of wood frogs (*Rana sylvatica*). *Am. J. Physiol.* **257**, R1046-R1049.
- Layne, J. R., Jr, Costanzo, J. P. and Lee, R. E., Jr (1998). Freeze duration influences postfreeze survival in the frog *Rana sylvatica*. *J. Exp. Zool.* **280**, 197-201.
- Lee, R. E. (2010). A primer on insect cold-tolerance. In *Low Temperature Biology of Insects* (ed. D. L. Denlinger and R. E. Lee), pp. 3-24. New York, NY: Cambridge University Press.
- Lee, R. E., Jr and Costanzo, J. P. (1998). Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *Annu. Rev. Physiol.* **60**, 55-72.
- MacArthur, D. L. and Dandy, J. W. T. (1982). Physiological aspects of overwintering in the boreal chorus frog (*Pseudacris triseriata maculata*). *Comp. Biochem. Physiol.* **72A**, 137-141.
- Macmillan, H. A. and Sinclair, B. J. (2011). Mechanisms underlying insect chill-coma. *J. Insect Physiol.* **57**, 12-20.
- Mazur, P. (2004). Principles of cryobiology. In *Life in the Frozen State* (ed. B. J. Fuller, N. Lane and E. E. Benson), pp. 3-65. Washington, DC: CRC Press.
- Middle, L. B. and Barnes, B. M. (2001). Overwintering physiology of the wood frog, *Rana sylvatica*, in Interior Alaska. *Am. Zool.* **41**, 1526-1527.
- Muir, T. J., Costanzo, J. P. and Lee, R. E., Jr (2008). Metabolic depression induced by urea in organs of the wood frog, *Rana sylvatica*: effects of season and temperature. *J. Exp. Zool.* **309**, 111-116.
- Muir, T. J., Costanzo, J. P. and Lee, R. E. (2010a). Brief chilling to subzero temperature increases cold hardiness in the hatchling painted turtle (*Chrysemys picta*). *Physiol. Biochem. Zool.* **83**, 174-181.
- Muir, T. J., Costanzo, J. P. and Lee, R. E., Jr (2010b). Evidence for urea-induced hypometabolism in isolated organs of dormant ectotherms. *J. Exp. Zool.* **313A**, 28-34.
- Packard, G. C., Lang, J. W., Lohmiller, L. D. and Packard, M. J. (1997). Cold tolerance in hatchling painted turtles (*Chrysemys picta*): supercooling or tolerance for freezing? *Physiol. Zool.* **70**, 670-678.
- Rexer-Huber, K. M. J., Bishop, P. J. and Wharton, D. A. (2011). Skin ice nucleators and glycerol in the freezing-tolerant frog *Litoria ewingii*. *J. Comp. Physiol. B* **181**, 781-792.
- Rubinsky, B., Wong, S. T. S., Hong, J.-S., Gilbert, J., Roos, M. and Storey, K. B. (1994). <sup>1</sup>H magnetic resonance imaging of freezing and thawing in freeze-tolerant frogs. *Am. J. Physiol.* **266**, R1771-R1777.
- Sait, R. W. (1963). Delayed inoculative freezing of insects. *Can. Entomol.* **95**, 1190-1202.
- Schiller, T. M., Costanzo, J. P. and Lee, R. E. (2008). Urea production capacity in the wood frog (*Rana sylvatica*) varies with season and experimentally induced hyperuremia. *J. Exp. Zool.* **309A**, 484-493.
- Schmid, W. D. (1982). Survival of frogs in low temperature. *Science* **215**, 697-698.
- Sinclair, B. J., Addo-Bediako, A. and Chown, S. L. (2003). Climatic variability and the evolution of insect freeze tolerance. *Biol. Rev. Camb. Philos. Soc.* **78**, 181-195.
- Storey, K. B. (2004). Vertebrate freeze tolerance: role of freeze-responsive gene expression. In *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application* (ed. B. M. Barnes and H. V. Carey), pp. 299-306. Fairbanks, AK: Institute of Arctic Biology, University of Alaska Fairbanks.
- Storey, K. B. (2006). Reptile freeze tolerance: metabolism and gene expression. *Cryobiology* **52**, 1-16.
- Storey, K. B. and Storey, J. M. (1988). Freeze tolerance in animals. *Physiol. Rev.* **68**, 27-84.
- Swanson, D. L. and Graves, B. M. (1995). Supercooling and freeze intolerance in overwintering juvenile spadefoot toads (*Scaphiopus bombifrons*). *J. Herpetol.* **29**, 280-285.
- Vali, G. (1995). Principles of ice nucleation. In *Biological Ice Nucleation and Its Applications* (ed. R. E. Lee, G. J. Warren and L. V. Gusta), pp. 1-28. St. Paul, MN: American Phytopathological Society Press.
- Voituron, Y., Mouquet, N., de Mazancourt, C. and Clobert, J. (2002). To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms. *Am. Nat.* **160**, 255-270.
- Walters, K. R., Jr, Serianni, A. S., Voituron, Y., Sformo, T., Barnes, B. M. and Duman, J. G. (2011). A thermal hysteresis-producing xylomannan glycolipid antifreeze associated with cold tolerance is found in diverse taxa. *J. Comp. Physiol. B* **181**, 631-640.
- Weisrock, D. W. and Janzen, F. J. (1999). Thermal and fitness-related consequences of nest location in painted turtles (*Chrysemys picta*). *Funct. Ecol.* **13**, 94-101.
- Zimmerman, S. L., Frisbie, J., Goldstein, D. L., West, J., Rivera, K. and Krane, C. M. (2007). Excretion and conservation of glycerol, and expression of aquaporins and glyceroporins, during cold acclimation in Cope's gray tree frog *Hyla chrysoscelis*. *Am. J. Physiol.* **292**, R544-R555.