Physiological Ecology of Overwintering in Hatchling Turtles

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ABSTRACT

Temperate species of turtles hatch from eggs in late summer. The hatchlings of some species leave their natal nest to hibernate elsewhere on land or under water, whereas others usually remain inside the nest until spring; thus, post-hatching behavior strongly influences the hibernation ecology and physiology of this age class. Little is known about the habitats of and environmental conditions affecting aquatic hibernators, although laboratory studies suggest that chronically hypoxic sites are inhospitable to hatchlings. Field biologists have long been intrigued by the environmental conditions survived by hatchlings using terrestrial hibernacula, especially nests that ultimately serve as winter refugia. Hatchlings are unable to feed, although as metabolism is greatly reduced in hibernation, they are not at risk of starvation. Dehydration and injury from cold are more formidable challenges. Differential tolerances to these stressors may explain variation in hatching overwintering habits among turtle taxa. Much study has been devoted to the cold-hardiness adaptations exhibited by terrestrial hibernators. All tolerate a degree of chilling, but survival of frost exposure depends on either freeze avoidance through supercooling or freeze tolerance. Freeze avoidance is promoted by behavioral, anatomical, and physiological features that minimize risk of inoculation by ice and ice-nucleating agents. Freeze tolerance is promoted by a complex suite of molecular, biochemical, and physiological responses enabling certain organisms to survive the freezing and thawing of extracellular fluids. Some species apparently can switch between freeze avoidance or freeze tolerance, the mode utilized in a particular instance of chilling depending on prevailing physiological and environmental conditions.


seemingly narrow focus of this project may surprise the casual reader, but neonates differ biologically from other age classes in so many respects as to warrant special treatment. Although the literature pertaining to overwintering in adult turtles has been comprehensively reviewed (Ultsch, '89, 2006), a comparable work focused on hatchlings is lacking.

A secondary objective is to call attention to weaknesses and gaps in our current knowledge and suggest directions for future study. Much of the information about the winter ecology of hatchling turtles is derived from anecdotal reports and casual field observations, as few field studies have been published. On the other hand, our knowledge of their winter physiology is based almost exclusively on the results of laboratory studies. Although many turtle species have temperature-dependent sex determination (Ewert et al., '94), gender-related distinctions in physiology and cold-hardiness responses are virtually unexplored. A preponderance of the literature concerns temperate North American turtles, certain species of which are disproportionately represented. This article necessarily reflects these biases.

Finally, we offer the following comments about the taxonomic nomenclature used in this article. Recent advances in molecular-based genetics and morphometric analyses have prompted taxonomists to reclassify and rename various turtles (e.g., over 50 new terminal taxa have been proposed in the last 15 yr); consequently, turtle nomenclature is perpetually dynamic and sometimes contentious (Iverson et al., 2007; Turtle Taxonomy Working Group, 2007). Given this state of flux, and being mindful that the primary utility of taxonomic nomenclature is name recognition (Smith and Chiszar, 2006), we used groupings and names that have predominated in the literature for at least the past 5 yr.

**OVERWINTERING ECOLOGY**

**Patterns of nest emergence**

A female turtle lays her eggs on land, depositing them in an excavation and covering them with substrate, and then moves on. In temperate regions, turtles nest one or more times from May to July, choosing warm, unshaded sites conducive to embryonic development (Christens and Bider, '87; Schwarzkopf and Brooks, '87; Rozycki, '98). Eggs usually hatch 2–3 months later, in late summer or early autumn, although unseasonably cool weather may delay or even prevent hatching (Ewert, '85; Packard and Packard, '88). Neonates apparently remain in the nest for some time after hatching, but ultimately they leave, either by dislodging the overlying soil, or “nest plug,” and ascending through the opening or by descending through the floor into the soil column below. Nest evacuation can occur before or after winter; thus, post-hatching behavior dictates many aspects of the hibernation ecology and physiology of hatchling turtles.

Physical and biological factors stimulating turtles to leave their nests are of considerable interest to chelonian ecologists, although there is little consensus about which of these are of greatest importance. Nest emergence sometimes coincides with rain events (Hammer, '69; Moll and Legler, '71; Nagle et al., 2004), suggesting that egress is triggered by a rapid increase in soil moisture. Precipitation could also stimulate emergence by softening the soil (Goode and Russell, '68; DePari, '96) or by flushing carbon dioxide from the nest and delivering the oxygen needed for locomotor activity (Prange and Ackerman, '74).

Temperature, a particularly labile environmental cue, is another potential inducer. With the advent of spring weather, reversal of the temperature gradient in the soil column could stimulate emergence in hatchlings (Bleakney, '63; Tucker, '99) as it does in adult turtles (Grobman, '90; Crawford, '91b) and also in squamates (Sexton and Marion, '81; Etheridge et al., '83; Costanzo, '86). Thermal cues, such as rising spring temperatures, could help synchronize emergence activities of hatchling turtles (Gibbons and Nelson, '78; Blouin-Demers et al., 2000). In a laboratory study of hatchling red-eared sliders (Trachemys scripta), longer periods of cold exposure hastened emergence from artificial nests on rewarming (Thomas, '99). Once a particular temperature threshold is reached, further chilling or warming may trigger nest emergence (Moran et al., '99; Nagle et al., 2004).

Environmental cues are mediated by physiological mechanisms that proximally determine behavioral responses. Unfortunately, studies addressing physiological regulation of nest emergence behavior (or any other post-hatching behavior) in hatchling turtles are lacking. Comparing the endocrine status of emerging and still-dormant hatchlings, and investigating populations that exhibit both fall emergence and spring emergence, might prove rewarding. In his study of Chrysemys picta, DePari ('96) found that hatchlings emerging in fall had shorter plastrons but weighed the same and had similar amounts of
residual yolk as turtles that overwintered inside their nests. Working with the same species, Costanzo et al. (2004) found no difference in the blood and somatic characteristics between fall-emerging and spring-emerging individuals, save for a higher lipid content in the former. However, neither study tested hatchlings for hormonal changes that might trigger emergence.

Endogenous factors could be important in nest emergence behavior. Lindeman (’91) found remarkable uniformity in the time interval (~300 d) between oviposition and hatching emergence and posited whether an internal “clock” cues emergence activity. Internal timing mechanisms have been implicated in stimulating emergence in turtles that vacate nests soon after hatching (Moran et al., ’99) and could also trigger emergence of hatchlings following hibernation, as they apparently do in some squamates (Drda, ’68; Garrick, ’72; Weatherhead, ’89; Lutterschmidt et al., 2006). Future research may ultimately show that the driving force for hibernation emergence is an interaction between extrinsic and intrinsic influences (Blouin-Demers et al., 2000).

Timing of nest emergence varies among taxa, populations, and even among siblings sharing the same nest. Emergence patterns in temperate species are typified by fall emergence, wherein hatchlings exit the nest before winter and hibernate elsewhere, and delayed emergence, wherein hatchlings remain inside the natal nest until the following spring. Although this dichotomy is ingrained in the literature, problems arise if it is taken too literally. First, the idiom “delayed emergence” invites ambiguity because even fall-emerging turtles do not exit the nest immediately after hatching, but rather remain in situ for at least several days, during which time they unfold their shells and absorb their external yolk sac (Burger, ’76a; Swingland and Coe, ’78; Christens, ’90; Díaz-Paniagua et al., ’97). Emergence latency can occur even among turtles that emerge in fall and hibernate outside the nest (Moll and Legler, ’71; Díaz-Paniagua et al., ’97; Mitrus and Zemanek, ’98). In a Michigan study, for example, snapping turtles (Chelydra serpentina) hatched in late August but left the nest about 2 months later (Sexton, ’57). In addition, the distinction between fall emergence and delayed (spring) emergence can be ambiguous, especially when considering species (and populations) of southerly climes. In northwestern Florida, for example, moderate temperatures permit hatchlings to emerge from summer through the following spring, even in midwinter (Aresco, 2004). By contrast, in cool-temperate regions the emergence pattern is a punctuated continuum, probably because nest egress is hampered by seasonal cold. In this case, “spring emergence” is a more appropriate idiom than “delayed emergence.”

Although it is tempting to categorize turtle species with respect to emergence timing, it is important to note that whereas nest emergence behavior apparently varies little in some species, in others it is quite plastic and either or both modes may be used, depending on the population and, perhaps, particular environmental and ontogenetic circumstances. Overwintering in the nest purportedly is an adaptation for survival in the northern portions of a species’ range (Carr, ’52; Congdon and Gibbons, ’85), although it certainly occurs in more southern temperate and subtropical areas (Cagle, ’50; Jackson, ’94; Buhlmann, ’98; Buhlmann and Coffman, 2001; Morjan and Stuart, 2001; Aresco, 2004; Swarth, 2004). Ecological and evolutionary implications of remaining in the natal nest during winter is a topic of broad interest (Wilbur, ’75a,b; Gibbons and Nelson, ’78) and will be addressed below.

Timing and patterns of nest emergence, both among nests at a given locale and among siblings sharing the same nest, may have marked survival consequences that are incompletely understood. Synchronization of life-stage transitions and other phenological phenomena often represent a predator-swamping strategy that increases offspring survival (Sweeney and Vannote, ’82; Rutberg, ’87; Ims, ’90; but see Tucker et al., 2008). For a clutch of hatching turtles, emerging in unison not only obviates mass predation but also facilitates egress and reduces each individual’s energetic cost (e.g., Brännäs, ’95; Spencer et al., 2001). Emergence synchrony may improve survival because hatchlings remaining even briefly within a breached nest are imperiled if the open chamber attracts parasites and predators (Burger, ’77; Congdon et al., ’83b, ’87, 2000; Christiansen and Gallaway, ’84; Christens and Bider, ’87; McGowan et al., 2001) or heightens the risk of dehydration and/or cold stress (Nagle et al., 2004; Baker et al., 2006). Late-emerging hatchlings may lag in somatic growth and poorly compete for limited resources (e.g., Mason and Chapman, ’65; Einum and Fleming, 2000) with potential fitness consequences manifested later in life (Yearsley et al., 2004). On the other hand, asynchrony in life-stage transitions also can be advantageous (e.g., Clark and Wilson, ’81; Théron and Combes, ’95) and can

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represent a risk-spreading strategy to increase fitness in an unpredictable and harsh environment (Danforth, '99; Thumm and Mahoney, 2002). Asynchronous emergence of turtle siblings could, in principle, minimize costs of remaining inside the natal nest longer than necessary (Hays et al., '92; Houghton and Hays, 2001). In addition, selection may favor asynchronous emergence if predators are attracted to high densities and large groups of hatchlings (Glen et al., 2005).

Emergence of turtle hatchlings from nests at a given locale may occur virtually simultaneously or over a surprisingly lengthy term. For hatchlings that overwintered inside the natal nest, the emergence period reportedly lasts from a few days to several weeks (Hartweg, '44; Lindeman, '91; DePari, '96; Díaz-Paniagua et al., '97; Tucker, '99; Bjurlin and Bissonette, 2004; Nagle et al., 2004), but could be even longer. For example, at one study site in northern Indiana, hatchling *Chrysemys picta marginata* that overwintered in their nests emerged from early March through late April (Fig. 1).

Proximate causes of variation in emergence timing are unknown, but probably relate to spatial variation in environmental factors (e.g., temperature, moisture) that serve as cues and/or facilitate escape from the confines of the hibernaculum. In turn, these factors are influenced by the physical attributes of individual nests, such as depth within the soil column, soil characteristics, drainage, slope, and aspect. Emergence behavior could also be influenced by an endogenous timing mechanism (Lindeman, '91), but this idea has not been rigorously tested. Some investigators have questioned whether clutches hatching relatively late in summer or in early fall, perhaps as a consequence of retarded development or delayed oviposition, also emerge late from their nests in autumn (Parren and Rice, 2004) or even the following spring (Ernst et al., '94). However, there is little empirical support for this relationship (e.g., Díaz-Paniagua et al., '97).

Assuming that emergence requires strenuous physical activity, inter-clutch variation in emergence timing could reflect differences in hatchling body size and physiological condition, or even size of the sibling group, if social facilitation is important to egress (Carr and Hirth, '61; Moran et al., '99). Stress imposed by dehydration or (for terrestrial hibernators) extreme cold could impair neurobehavioral function, delaying nest emergence (see Hartley et al., 2000). Similarly, following especially severe winters, adult painted turtles (*C. picta*) resume behavioral activity relatively late in spring, presumably because they require an extended recovery period (Crawford, '91b). This idea has not been experimentally tested; however, Lindeman ('91) observed that surviving hatchlings in clutches suffering high winter mortality (a reasonable proxy for stress) tended to emerge later than hatchlings in clutches with higher survival.

Clutchmates do not necessarily emerge from their nest en masse, but may exit in small groups or individually over protracted periods. Relatively little study has been devoted to this phenomenon, in part owing to the technical challenge of concurrently monitoring multiple nests (see Doody and Georges, 2000). Consequently, some authors have presumed that clutchmates emerge more or less in unison, once the first hatching has appeared (Christens and Bider, '87; Tucker, '97, '99). However, several reports attest that individuals emerge sporadically over many days or even weeks (Burger, '77; Hays et al., '92; Butler and Graham, '95; Díaz-Paniagua et al., '97; Houghton and Hays, 2001; Bjurlin and Bissonette, 2004). In the extreme, some individuals emerge in fall, the remainder of the clutch overwintering in the open nest and, if able to survive, emerging in spring (Costanzo et al., 2004; Nagle et al., 2004; Swarth, 2004; Baker et al., 2006; Carroll and Ultsch, 2007). This pattern, reported for several taxa, is probably uncommon, but underscores the plasticity in emergence behavior among hatching turtles in at least temperate regions.

Factors influencing timing and patterns of emergence of siblings are as yet undetermined. Variation in body size and/or physiological condition among clutchmates could contribute to asynchrony (Glen et al., 2005), although findings to the contrary have been published (Hays et al.,...
of asynchrony. It is possible that clutchmates receive emergence cues at different times; indeed, emergence asynchrony tends to increase with thermal variation inside nests (Houghton and Hays, 2001). Environmental heterogeneity within the nest could also cause siblings to become differentially impacted by stressors (e.g., cold, freezing, dehydration, and hypoxia) that potentially impair neurobehavioral function. Because motor support systems are sensitive to oxygen availability, solute and water balance, ion gradients, and acid–base status (Miller et al., ’87; Lutz and Storey, ’97; Finkler, ’99; Jackson, 2002), such stress could delay or even prevent emergence of afflicted individuals. This notion has not been formally tested, but it is noteworthy that Glen et al. (2005) found that nests with higher numbers of dead hatchlings also had longer emergence durations and higher levels of asynchrony.

**Overwintering habits and habitats**

In recent years, interest in chelonian conservation and mechanisms of cold hardiness has generated a wealth of new information about the winter ecology of hatchling turtles. Still, there remain substantial voids in our knowledge of the hibernation habits of even common species, and especially of those that hibernate aquatically (Ultsch, 2006). This paucity probably stems from the difficulty in monitoring large samples of nests, and in locating hatchlings after they disperse from the nesting site. Dispersal behavior has been studied by tracking movements of hatchling turtles treated with fluorescent dye (e.g., Butler and Graham, ’93; Tuttle and Carroll, 2005), but this method has significant limitations. Improvements in radiotelemetry and other tracking techniques would enable investigators to monitor hatchling behavior for sufficiently long periods, providing a more thorough understanding of their winter habits and habitat requirements. The recent study of hatchling *C. serpentina* by Ultsch et al. (2008) is a case in point.

Naturalists have long presumed that the hatchlings of terrestrial turtles hibernate on land and those of freshwater turtles passed the winter under water. However, field investigations over the last several decades have revealed that hibernacula used by neonatal turtles are variable and diverse, and sometimes distinct from those used by other age classes.

Hatchlings of terrestrial species tend to hibernate on land, either inside the nest or outside, some burrowing into the soil column to hibernate at great depths (Doroff and Keith, ’90; Costanzo et al., ’95b; Converse et al., 2002). Hatchlings of some aquatic species also overwinter in the natal nest, although many apparently hibernate under water. Published reports characterizing the aquatic refugia of hatchlings are lacking (but see Ultsch et al., 2008). Hatchlings and young turtles seemingly prefer shallow-water habitats for feeding and predator avoidance (Hart, ’83; Congdon et al., ’92; Pappas and Brecke, ’92), but whether or not they also hibernate in these locations remains to be determined. Conventional wisdom maintains that hatchlings probably hibernate in proximity to older conspecifics, which are known to use burrows or other cavities, take refuge under cover objects, or settle on or beneath the substratum surface. However, recent findings that hatchlings are relatively intolerant of hypoxic submergence (Reese et al., 2004b; Dinkelacker et al., 2005b) raise questions about their ability to overwinter in certain aquatic microenvironments. This liability may limit the availability of suitable hibernacula or could suggest that mortality rates are especially high in hatchlings. On the other hand, hatchlings might preferentially use lotic systems or the more oxygenated regions of lentic habitats, such as areas of upwelling or inflowing water, air pockets beneath the ice cap, or the land/water interface. Careful field research is needed to resolve these questions.

Aquatic species that leave their nest before winter do not necessarily hibernate in water. In a radiotelemetry study on Long Island, NY, hatchling *C. serpentina* wandered through various habitats and encountered a pond, but instead chose to overwinter in nearby spring seeps (Ultsch et al., 2008). Blanding’s turtles (*Emydoidea blandingii*) typically emerge from their nests in late summer and wander considerably, apparently avoiding water (Standing et al., ’97; McNeil et al., 2000), before overwintering. Their hibernation habits are as yet unknown, although some authors speculate that they overwinter in waterlogged soils near the margins of ponds or wetlands (Dinkelacker et al., 2004). In an estuarian species, the diamondback terrapin (*Malaclemys terrapin*), some hatchlings vacate the nest, briefly inhabit the upper intertidal zone, and then return to land to hibernate in shallow burrows (Draud et al., 2004).

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Hibernation in the natal nest

A persistent question pondered by turtle researchers is why the hatchlings of some species overwinter in the nest whereas others do not. A related question is why any hatchling would overwinter in the nest at all. Gibbons and Nelson ('78) addressed the latter, reporting that, in South Carolina, several aquatic species evidently spend their first winter on land, presumably inside the nest. Their synthesis consolidated a host of early, mostly anecdotal reports, emphasizing that hibernation of neonates in the natal nest occurs worldwide and in at least a dozen genera and five families. In addition, this seminal report offered an evolutionary explanation for variation in emergence patterns both within and among species. Subsequent authors have posited myriad explanations for why hatchlings overwinter in the nest, some supposing that the behavior is a passive response, the ultimate consequence of adverse biological or environmental conditions hampering fall emergence, and others regarding it as a facultative strategy that increases offspring fitness.

Passive response hypotheses

One popular hypothesis maintains that hatchling turtles pass the winter inside the natal nest because they are physically incapable of breaching the hardened nest plug until freezing/thawing and vernal rains have softened the soil (Cagle, '44; Hartweg, '44; Legler, '54; Ernst, '66; Tinkle et al., '81; DePari, '96). Support for this idea comes from observations that drought precedes overwintering in the nest of species (populations) that typically emerge in autumn, and that emergence events in spring often coincide with or closely follow rainfall. For example, emergence of hatchling *C. serpentina* is reportedly facilitated by annual rains (Hammer, '69), whereas dry, hardened soil apparently impedes egress and causes them to remain inside nests during winter (Ernst, '66). In northern New Jersey, DePari ('96) found a strong association between nest emergence timing of *C. picta* and characteristics of the nesting soil. Hatchlings occupying natural and artificial nests constructed in friable soils (sands) were more likely to emerge in autumn than turtles in heavier or wetter soils, suggesting that the ability to overcome physical barriers to egress can influence emergence timing.

Nest entrapment could explain interspecific variation in overwintering behaviors of hatchling turtles. For example, if breaching the nest plug is facilitated by sibling cooperation, then emerging in autumn may be easier and more consistent in species (e.g., *C. serpentina*) that produce relatively large clutches (DePari, '96). On the other hand, fall emergence is the norm in some species that invariably produce few young (Gibbons and Nelson, '78). Perhaps for these species social facilitation is not required because their nests have little overburden (e.g., *Sternotherus* spp.), their nests are constructed in friable soils (e.g., *Terrapene* spp.), or they are especially adept at burrowing into the soil column (e.g., *Kinosternon* spp.).

Contrary to the arguments listed above, some observations suggest that entrapment is not a primary cause of overwintering inside the natal nest. Late summer rains that soften the soil and even erode the nest plug do not necessarily stimulate emergence (Hartweg, '44; Sexton, '57; Bleakney, '63; Gibbons and Nelson, '78; DePari, '96). Furthermore, hatchlings of some species routinely overwinter inside nests constructed in friable soils from which egress should be relatively easy (Costanzo et al., 2004; Baker et al., 2006). Hatchlings may be forced to overwinter inside their nest at least occasionally, but, as a general explanation, the concept of entrapment is unsatisfying.

Heat is an important resource not only for embryonic development and hatching but also for nest emergence activities. Accordingly, overwintering in the nest could result if fall emergence is hampered by the onset of cool weather. Spring emergence seems to coincide with a seasonal reversal of the vertical thermal gradient, suggesting that turtles are thermotactic (Bleakney, '63; Tucker, '99). Therefore, if, in autumn, the soil strata above the nest become cooler than those below, emergence cues would be lacking. In addition, the hindering effect of cold on locomotor function could directly prevent turtles from leaving their nests. Thermal insufficiency may account for the failure of some clutches of obligate aquatic hibernators to emerge in autumn (Sexton, '57; Breckenridge, '60; Obbard and Brooks, '81a; Parren and Rice, 2004) or even to hatch (Buech et al., 2004). On the other hand, nest temperatures at the time of hatchling emergence are commonly even lower in spring than in autumn (Gibbons and Nelson, '78; Holte, '88; DePari, '96) therefore, the role of cold, per se, in inhibiting fall emergence is unclear.

Yet another explanation is that hatchlings are developmentally immature and unprepared to leave the nest in autumn. Thermal and hydric factors strongly influence hatchling phenotype and
fitness (Packard and Packard, '88; Deeming, 2004a), and suboptimal incubation conditions could produce immature hatchlings. Immaturity at hatching also could result from delayed oviposition or, at high latitudes, brevity of the summer. In northern populations of *C. picta*, hatching may not occur until autumn (Costanzo et al., 2004). Declining soil temperatures apparently retard embryonic development and ultimately hamper fall emergence of hatchlings of several species (Bleakney, '63; Gibbons and Nelson, '78; Mitrus and Zemanek, '98; Waye and Gillies, '99). In some populations of the European pond turtle (*Emys orbicularis*), aquatic hibernation is the norm, but late-hatching clutches may remain in the nest throughout winter, perhaps because they are immature and are unable to respond to environmental emergence cues (Drobenkov, 2000). On the other hand, in temperate regions, even early-hatching clutches sometimes overwinter inside the nest (Gibbons and Nelson, '78; Jackson, '94; Morjan and Stuart, 2001). Clearly, developmental immaturity is not a universal factor influencing emergence timing.

Deviation from the routine hibernation habit undoubtedly occurs in hatchlings of virtually every species. For obligate aquatic hibernators, remaining inside the natal nest during winter could stem from failure of autumnal emergence cues to materialize or to be detected (or acted on) by hatchlings. This scenario could explain the odd case reported by Obbard and Brooks ('81a), where between 1976 and 1979 in Algonquin Park, Ont., 62 of 104 *C. serpentina* clutches (59.6%) producing viable hatchlings overwintered inside the nest (with lethal consequences), rather than emerging in autumn. Conversely, in species whose hatchlings usually overwinter terrestrially, fall emergence could be triggered by adverse conditions, such as flooding or degradation of the nest plug or chamber. For example, although ornate box turtles (*Terrapene ornata*) usually spend their first winter in the soil column beneath the nest chamber, clutches hatching in particularly shallow nests sometimes emerge in fall and hibernate elsewhere (Converse et al., 2002). In some populations of *C. picta*, plasticity in nest emergence behavior apparently is tied to local weather and soil conditions (DePari, '96).

**Adaptive response hypotheses**

A popular alternative hypothesis for why turtles hibernate in the natal nest states that deferring emergence until spring confers special benefits that increase the probability of winter survival. Overwintering within the nest carries its own risks, such as death from flooding, predation, dehydration, energy depletion, or extreme cold, and may eliminate the opportunity for early feeding and accelerated growth. However, Gibbons and Nelson ('78) argued that, at least in some circumstances, these costs could be offset by certain gains. For example, overwintering in the nest would be advantageous if hatchlings emerging in spring entered an environment in which thermal and food resources were increasing, rather than decreasing, as would be the case in autumn. According to this argument, natural selection should favor deferred emergence from a "proven sanctuary" until environmental cues signal that hatchlings will enter a resource-rich environment favoring rapid somatic growth (Gibbons and Nelson, '78; Mitchell, '88).

From this reasoning, one would expect overwintering inside the nest to be commonplace, if not obligatory (see Carr, '52), for hatchlings of northern species. However, there may be more species of northern turtles whose hatchlings emerge in fall (e.g., *C. serpentina*, *E. blandingii*, *Clemmys guttata*, *Clemmys* [*Glyptemys*] *insculpta*, *Apalone spinifera*, some *E. orbicularis*) than there are of species whose hatchlings overwinter in the nest (e.g., *C. picta*, *Graptemys geographic* *a*, *T. scripta*, some *E. orbicularis*). Perhaps the thesis of Gibbons and Nelson ('78) applies generally over much of the temperate region, but does not apply in northern regions because there the perils of remaining inside the nest are too severe. The fact that the facultative nest hibernator, *C. picta*, commonly overwinters inside the nest, even in the northernmost reaches of its distributional range (e.g., Woolverton, '63; St. Clair and Gregory, '90; Rozycki, '98), suggests that this behavior is advantageous if the associated challenges can be overcome.

Overwintering inside the nest could constitute a means of reducing predation risk during a period of limited opportunity for somatic growth. This argument was advanced for *C. picta* (Wilbur, '75a,b), but certainly would apply to other temperate species. Hatchlings remaining in the nest during winter may be relatively safe, as depredation of turtle nests generally diminishes with time after oviposition (Burger, '77; Tinkle et al., '81; Christens and Bider, '87; Rozycki, '98). Remaining

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1Recommended by the Committee on Standard English and Scientific Names (Iverson et al., 2008).
in situ until spring eliminates the need to disperse overland at the time predator populations have reached a seasonal peak and some migratory predators have concentrated in staging areas. Additionally, after making the trek to water, hatchlings would become exposed to both resident and migratory predators, such as wading birds, which are feeding heavily in preparation for winter. Little is known about predation pressure on hatchling turtles in aquatic habitats, in part owing to the difficulty of tracking the fate of this cohort (e.g., Brooks et al., '91). Hatchlings of many aquatic turtles exhibit effective antipredator behavior and aposematic coloration, and probably are not preyed on heavily by fish (Semlitsch and Gibbons, '89; Britson and Gutzke, '93). More research in this area would permit comparison of the cost/benefit relationships of overwintering in the nest and in aquatic habitats.

Another potential benefit of passing the winter inside the "proven sanctuary" is that protection is afforded to turtles that have hatched, but are not morphologically or physiologically mature enough to survive outside the nest. These animals would have additional time to complete development in a relatively safe environment where demands for performance (feeding and digestion, evading predators, habitat selection, etc.) are relatively few. Neonatal C. picta dive and swim poorly, perhaps owing to high buoyancy imparted by their large internalized yolk sac (T. Muir, personal communication). Turtles consume much of their yolk during winter and presumably are more adroit when they finally enter water. Accordingly, species whose hatchlings provision their eggs with relatively large amounts of yolk also tend to defer emergence until spring (Congdon et al., '83c; Congdon and Gibbons, '85, '90; Rowe et al., '95; Nagle et al., '98; Costanzo et al., 2000b); however, this association may be derived from additional (or other) factors.

From a physiological perspective, overwintering within the nest may be an adaptive response that obviates problems associated with submergence in cold, hypoxic water. It may be more favorable energetically if turtles in nests substantially reduce their metabolic demands. Intuitively, this may be the case if nesting soil, by virtue of its lower specific heat, cools more quickly than water, and if prevailing winter temperatures are lower inside nests than within aquatic refugia (Fig. 2). However, few investigations have addressed the physiology of aquatic hibernation in hatchling turtles (Finkler et al., 2002; Reese et al., 2004b; Dinkelacker et al., 2005a,b) and none have compared the energetic costs of overwintering in water with those of overwintering on land.

**Ecological and evolutionary implications**

As mentioned above, one particularly vexing question is why some species hibernate in the nest in a given area, particularly in the northern parts of their ranges, whereas other species do not. If hibernating in the nest confers a fitness advantage relative to emerging in autumn for any of the reasons listed above, then, intuitively, all species ought to exhibit this behavior. Several hypotheses can be advanced to explain why this is not the case.

First, perhaps only species with strictly northern distributions have evolved a life history that includes overwintering of hatchlings inside the nest. For example, with the exception of the southern subspecies, C. picta is basically a northern taxon whose hatchlings typically hibernate in the nest, whereas musk turtles, with the exception of one species (Stenotherus odoratus), are basically a southern group whose hatchlings overwinter elsewhere. On the other hand, two species of map turtles (genus Graptemys), a predominantly southern group, range into cold climates and their hatchlings do overwinter inside the nest. A telling argument contradicting this hypothesis is that several species (e.g., C. guttata, C. insculpta,
E. blandingii) that are closely related, basically northern forms, have hatchlings that apparently routinely hibernate outside the nest.

A second hypothesis is that overwintering of hatchlings in the nest is restricted to species of certain phylogenetic groupings. Indeed, most of the species exhibiting this behavior are in the family Emydidae. On the other hand, most of the species inhabiting the United States and Canada are in this family. Moreover, a number of northern emydid species do not, and perhaps cannot, successfully overwinter inside the natal nest. It is noteworthy that, among emydids, most of those successfully overwinter inside the natal nest. It is not all emydids use the same cold-hardiness strategies (see below).

Another hypothesis is that only species in certain morphological or physiological groupings have hatchlings that overwinter in the nest. In northern environs, all such species must be adapted to cope with exposure to potentially injurious cold and dehydration. Species whose primary strategy of cold hardiness is freeze tolerance share physiological adaptations that limit osmotic and anoxic damage to frozen cells and tissues, whereas species that avoid lethal freezing through supercooling (remaining liquid at temperatures below the equilibrium freezing/melting point) probably share behavioral and morphological traits that reduce the risk of contact inoculation. The integument is a particularly important mediator of both injurious freezing and evaporative water loss (EWL), and various species (e.g., C. serpentina, S. odoratus, and A. spinifera) that do not, and perhaps cannot, overwinter in the nest as hatchlings have a relatively large amount of skin exposed to their surroundings (Costanzo et al., 2001b). The implication of this relationship is that excessive dehydration and a greater propensity for inoculative freezing constrains nest overwintering behavior. On the other hand, species such as C. guttata, C. insculpta, and E. blandingii do not hibernate in the natal nest even though they have little exposed skin.

According to the aforementioned hypothesis, overwintering of hatchlings in the nest is the most favorable survival strategy, but is only used by species whose hatchlings can cope with the threats of freezing and/or desiccation. The alternative is to hibernate in places where these threats can be avoided, but other risks abound. Mortality during the first winter of life may well be higher among hatchlings that overwinter aquatically than among those that spend the first winter in the nest. Taking this point further, if a species has a high mortality not only during the egg stage, as all species do, but also during the initial winter because it hibernates aquatically, then it must compensate for this additional demographic bottleneck. One approach is to live long and/or have a high annual reproductive output. C. serpentina are long-lived, for example, and thus can persist in far northern populations (e.g., Algonquin Park, Ont.) in spite of very low annual reproductive success (Galbraith and Brooks, ’87; Brooks et al., ’91). Because they lay large clutches, an occasional successful year of reproduction is apparently enough to sustain the population.

If overwintering in the nest is more beneficial than hibernating elsewhere, then, lacking the challenges of severe cold and desiccation, hatchlings universally ought to do so. Accordingly, a species that does not overwinter in the nest in the northern portion of its range perhaps could do so in the more southern parts of its range, where cryoinjury is unlikely, if the nest environment were sufficiently humid. There is some support for this notion, but only in that the hatchlings of many species do commonly overwinter in the nest at low latitudes (Carr, ’52; Congdon and Gibbons, ’85), although it certainly occurs in more temperate areas (Cagle, ’50; Jackson, ’94; Buhlmann, ’98; Buhlmann and Coffman, 2001; Morjan and Stuart, 2001; Aresco, 2004; Swarth, 2004). There is no compelling evidence for a major reversal of hibernation habits with latitude for a given species. For example, hatchlings of A. spinifera, C. serpentina, S. odoratus, and T. carolina routinely emerge from the nest in autumn and overwinter outside the nest throughout their extensive geographical ranges. Additionally, although information for southern populations is meager, several species, including C. picta (Cagle, ’54) and T. scripta (Gibbons and Nelson, ’78; Jackson, ’94), which hibernate in the nest in the north apparently also do so in the south. Thus, a working hypothesis is that if a species can overwinter in the nest, then it will, and will do so throughout its geographic range. Clearly, however, such behavior is not a requirement for a species to be successful, or even to be competi-

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tive. *C. picta* and *C. serpentina* best exemplify this point: hatchlings of the former overwinter in the nest and those of the latter do not, yet both are abundant and wide-ranging species that coexist in very cold climates.

**Taxonomic and geographic patterns**

Overwintering habits and habitats of hatchling turtles are remarkably diverse, even among syntopic species (Christiansen and Gallaway, ’84; Costanzo et al., ’95b). For example, in the Sandhills region of westcentral Nebraska, hatchling *C. picta* hibernate within their natal nests, \( \approx 10 \text{ cm below ground} \); *T. ornata* and *Kinosternon flavescens* overwinter in sand dunes \( \geq 1 \text{ m beneath the nest chamber} \); and *A. spinifera* and *C. serpentina* emerge from their nests after hatching and move to ponds or streams for overwintering. As we will discuss below, the hibernation habits of a given species reflects its particular life-history traits and physiological tolerances to environmental extremes.

The hatchlings of some temperate species exhibit considerable variability in overwintering behavior on both regional and local scales. For example, hatchling *E. orbicularis* overwinter under water in central Poland (Mitrus and Zemanek, ’98), inside the natal nest in northeastern Germany (Andreas and Paul, ’98; Schnee- weiss et al., ’98), or in self-constructed burrows in southern Russia (Mazanaeva and Orlova, 2004). In Belarussian Polesye, near the northeastern edge of this species’ range, and in northeastern Ukraine, they can hibernate inside or outside the nest, depending on whether the eggs began incubation early or late, respectively (Drobenkov, 2000; Zinenko, 2004). The painted turtle of North America is also notable in this regard. Although overwintering inside the nest appears to predominate, autumnal emergence (and presumed aquatic hibernation) occurs with variable frequency within the same populations of all northern subspecies: *C. p. marginata* in Indiana (Costanzo et al., 2004), *C. p. picta* in New Jersey (DePari, ’96), Connecticut (Finneran, ’48), New Hampshire (Carroll and Ultsch, 2007), Pennsylvania (Ernst, ’71) and Maine (Rozycki, ’98), and *C. p. bellii* in New Mexico (C. Morjan, personal communication), Iowa (Christiansen and Gallaway, ’84), Nebraska (Costanzo et al., 2004), and Minnesota (Pappas et al., 2000). In British Columbia, near the northwestern edge of its geographic range, *C. p. bellii* either emerges from the nest in autumn (Waye and Gillies, ’99) or overwinters in situ (St. Clair and Gregory, ’90), attesting that the hibernation habit of this species is plastic, even in a severe environment. In this regard, *M. terrapin* is particularly interesting, as up to 30% of the nests on a given beach harbor hatchlings during winter (Roosenberg, ’94; Baker et al., 2006); young produced in the other nests seek winter quarters elsewhere (Auger and Giovannone, ’79).

In the final analysis, there is no definitive answer as to why hatchlings, especially those of aquatic species, overwinter inside the natal nest, nor is it clear why some species exhibit this behavior and others do not. The evidence does show, however, that species that can successfully hibernate in the nest will do so throughout their geographic range, and that the behavior occurs only incidentally in species that are not well suited to terrestrial hibernation. The adaptive significance of overwintering inside the nest remains equivocal.

**Species accounts**

Gibbons and Nelson (’78) earlier compiled the available information concerning the hibernation habits of the hatchlings of various turtle species. Much new information has subsequently become available, yet the contemporary literature lacks an updated account. We here summarize, for mostly North American species, the current state of knowledge pertaining to the overwintering habits and habitats of hatchling turtles, and briefly comment on the physiological and ecological implications of habitat selection.

**Terrestrial species**

*Gopherus* spp. (tortoises; Testudinidae): Of the three species of land tortoises in the United States (*G. agassizii*, *G. polyphemus*, and *G. berlandieri*), all are southern forms, as the northern limit of their range is southernmost Utah in the west and southernmost South Carolina in the east. Hatchlings of the first two species emerge from nests in autumn; unhatched eggs do not survive the winter (*G. agassizii*—Woodbury and Hardy, ’48; Turner et al., ’86; Rostal et al., ’94; Averill-Murray et al., 2002; Bjurlin and Bissone, 2004; *G. polyphemus*—Iverson, ’80; Landers et al., ’80; Wright, ’82; Martin, ’89; Butler et al., ’95; Butler and Hull, ’96; Epperson and Heise, 2003). *Gopherus berlandieri*, limited to southern Texas in the United States, probably also emerges in autumn, as the laboratory incubation period was 88–118 d for 13
eggs, with hatching occurring August–November (Judd and McQueen, ’80). Whether overwintering in the nest occurs in any tortoise is an open question, as it has been reported only once, for Testudo horsfeldii in Kazakhstan, Turkmenia, and Uzbekistan (Kuzmin, 2002).

Terrapene spp. (box turtles; Emydidae): These are terrestrial emydids with two species in the United States. Terrapene carolina (eastern box turtle) ranges from Florida to Texas north into New England and Michigan, and T. ornata (ornate box turtle) ranges across the Great Plains north to South Dakota. Eastern box turtle hatchlings emerge from nests in autumn throughout their range (Cooke, ’10; Cahn, ’33; Ewing, ’33; Allard, ’35, ’48; Conant, ’38; Smith, ’61; Minton, ’72; Iverson, ’77; Dundee and Rossman, ’89; Forsythe et al., 2004), although little is known about their hibernacula. Presumably they burrow below the frost line, as do adults (but see Costanzo and Claussen, ’90; Claussen et al., ’91). Some may also overwinter in or below the nest cavity (Ernst et al., ’94; Trauth et al., 2004), but such behavior appears rare. Hatchlings of T. ornata, however, routinely overwinter below the nest cavity at depths approaching 1 m (Doroff and Keith, ’90; Ernst et al., ’95b; Converse et al., 2002). The depth to which hatchings burrow suggests that they are attempting to stay below the frost line, although they do tolerate somatic freezing (Costanzo et al., ’95b, 2006; Dinkelacker et al., 2005b).

Aquatic species

C. serpentina (snapping turtle; Chelydridae): This species is wide ranging and common in the central and eastern United States and, with C. picta, has the most northerly range limit of North American turtles. Throughout its range, all reports of hatchlings observed leaving the nest or being collected in numbers are for autumn (Noble and Breslau, ’38; Hamilton, ’40; Pell, ’41; Cagle, ’44; Norris-Elye, ’49; Petokas and Alexander, ’60; Hammer, ’69; Punzo, ’75; Obbard and Brooks, ’81a,b; Congdon et al., ’87; Mitchell, ’94; Pappas et al., 2000; Hulse et al., 2001; Kolbe and Janzen, 2002; Carroll and Ultsch, 2007). It is widely accepted that these hatchlings overwinter aquatically, although nothing in the literature confirms this supposition. Some workers have found occasional hatchlings in spring that were presumed to have hibernated on land (Toner, ’40; Ernst, ’66; Minton, ’72; Congdon et al., ’87; Parren and Rice, 2004); however, because these individuals were not observed leaving a nest, it is uncertain where they spent the winter. They could have been waifs stranded on land that managed to survive the winter by burrowing, or simply relatively small turtles that were found on land after hibernating aquatically.

At high latitudes, hatchling C. serpentina will perish if they fail to emerge from their nests before winter, and pipping may not occur if cold weather arrives early enough to kill developing hatchlings. At Algonquin Park, Ont., very close to the northern limit of their range, Obbard and Brooks (’81a) found that of 257 clutches monitored, only 47 successfully emerged in autumn, and only one of the remaining nests had any survivors the following spring. This nest, which on excavation (May 5) was found to contain 16 live hatchlings, 11 dead hatchlings, and three infertile eggs, may have been buffered from cold by a snow bank formed by road plowing. Similarly, in North Dakota, Hammer (’69, ’72) found that hatchlings died in the nest if they failed to emerge before winter. Moreover, despite the usually high hatching success (93.5%), emergence rates were typically low (19.8%) and in some years no turtles left the nests. Why hatchlings in these northern populations did not leave the nest once they have left the egg remains an open question. In Michigan, some were found entangled in plant roots that prevented them from reaching the surface (J. Dusseau, personal communication). If hatching occurs late in autumn, low body temperatures may hamper their ability to dig out, or the overlying soil may freeze and become impenetrable.

In northern populations, hatchlings remaining in nests during winter are usually doomed, as they lack a well-developed capacity for freeze tolerance and cannot supercool extensively in the nest environment (Packard and Packard, ’90; Packard et al., ’93; Costanzo et al., 2001b, 2006; Dinkelacker et al., 2005b). However, in climates where freezing is not an issue, they perhaps could survive winter on land. Of particular note are the findings of J. Gibbons and J. Greene (unpublished data), who during 1978–1998 monitored a drift fence that encircled a bay in South Carolina. Of 45 hatchlings collected, four were captured moving toward the bay in February–April, presumably after overwintering on land, although not necessarily inside natal nests. In this population, autumnal nest emergence and aquatic hibernation is the rule, but successful overwintering on land may be possible.

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Hatchling *C. serpentina* on Long Island, New York and in southeastern New Hampshire were fitted with radiotransmitters and followed to their hibernation sites (Ultsch et al., 2008). On Long Island, all hatchlings initially moved to water, but later movements included terrestrial sojourns; those that could be located left the water and overwintered in spring seeps. In New Hampshire, hatchlings moved directly to nearby aquatic habitats after emergence and hibernated aquatically in root masses near banks.

*S. odoratus* (common musk turtle; Kinosternidae): This species ranges into southern Maine and Ontario in the north and to the Gulf Coast in the south. Most reports indicate that, through all its range, hatchlings emerge from natal nests in autumn and presumably overwinter aquatically (Risley, '33; Cagle, '42; Carr, '52; Minton, '72; Ernst, '86; Mitchell, '88).

Generally, it is easier to find hatchling turtles in fall, when most emerge from nests over 3–4 weeks, than in spring, when they may emerge over a period of months. This may be especially true for musk turtles, whose hatchlings are very small and rarely bask, and hence not readily observed. There are some anecdotal reports of finding hatchling musk turtles emerging from nests in spring (G. Folkerts [Alabama—emerging in spring from nests on beaver dams], E. Keiser [Mississippi], J. Weilbacher [Ohio], personal communications). Long-term monitoring of a drift fence encircling a South Carolina bay (as described in the section on *C. serpentina*) showed that of 60 hatchling musk turtles collected between 1969 and 2000, 11 were captured in March or June and 49 were captured in August or September (J. Gibbons and J. Greene, unpublished observations; see also Gibbons and Nelson, '78). In the Nebraskan Sandhills, hatchling *K. flavescens* descend over 0.5 m below the nest chamber to overwinter (Costanzo et al., '95b). This behavior presumably is necessary because they are susceptible to inculotive freezing (Costanzo et al., 2001b) and do not tolerate somatic freezing (Costanzo et al., '95b). It seems likely that *K. subrubrum* exhibits similar behavior in the northern part of its range.

The striped mud turtle, *Kinosternon baurii*, which inhabits the southeastern coastal plain from Virginia through Florida, has an atypical developmental pattern among turtles of the United States and Canada. Generally, embryonic development within the female proceeds to the late gastrula stage, and is arrested in the oviduct until the eggs are laid (Ewert, '85). *K. baurii* also exhibits embryonic diapause, a state of arrested development that usually can be terminated only by a period of prolonged chilling followed by rewarming (Ewert and Wilson, '96). Eggs laid in autumn, and at lower temperatures, are more likely to undergo diapause than eggs laid in spring or at relatively high temperatures. Because a given female may nest in autumn or spring (September–June, in Florida), the diapause is regulated environmentally, rather than genetically (Ewert and Wilson, '96). Eggs laid in spring probably hatch in summer and fall, but it is unknown how many of the hatchlings then overwinter inside the nest. Iverson ('77) found hatchlings outside the nest most frequently in January and March, but also in August, September, and December. Similarly, Mushinsky and Wilson ('92) reported predominantly spring emergence from nests, but also made some captures in October and December. Thus, there appears to be a minor emergence in fall and a major one in spring, perhaps with some individuals overwintering in nests as hatchlings and others as embryos.

Two southwestern/Mexican kinosternids (*K. sonoriense* and *K. integrum*) are found at high altitudes where winters can be cold (Degenhardt and Christiansen, '74). Both species exhibit embryonic diapause and, at least at low altitudes, late-stage embryos defer hatching until the following summer (Ewert, '91; Iverson, '99). Whether

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embryos can survive harsh winters at high altitudes (or else are forced to complete development in autumn) is an interesting question worthy of study.

_E. blandingii_ (Blanding’s turtle; Emydidae): One of the most northerly distributed of all chelonians, Blanding’s turtles reportedly leave the natal nest in autumn from their eastern limit in Nova Scotia to their western limit in Nebraska (Conant, ’38; Sexton, ’57; Bleakney, ’63; Minton, ’72; Graham and Doyle, ’78; Congdon et al., ’83b; Butler and Graham, ’95; Standing et al., ’97, ’99; McNeil et al., 2000; Pappas et al., 2000; Kolbe and Janzen, 2002). In Nova Scotia, Herman et al. (’95) found five apparently dormant hatchlings inside a nest in late fall, but it is uncertain what their fate would have been had they not been disturbed.

Although hatchlings commonly emerge from their nests in autumn, and their post-emergence behavior has received considerable study (Butler and Graham, ’95; Standing et al., ’97; McNeil et al., 2000; Pappas et al., 2000), it remains unclear whether they ultimately overwinter in water or on land. Some evidence suggests that they could successfully hibernate on land. For example, hatchlings are not especially diligent in heading for water after leaving the nest in autumn, some wandering on land for days, even if water was within 5 m (Standing et al., ’97, ’99; McNeil et al., 2000), who studied hatchings at the same location, made similar observations. Oddly, these hatchlings showed no directionality to open water and most avoided a lake near the nests. However, this behavior may be particular to this locale because no shallow-water bodies were nearby. Apparently hatchling _E. blandingii_ prefer shallow wetlands for overwintering and can travel considerable distances to such sites. For example, in Massachusetts, Butler and Graham (’95) tracked nine hatchlings that emerged from their nest. All went to water, but took up to 9 d and traveled a mean distance of 187 m to reach it. B. Butler and S. Smyers (personal communication) found that hatchlings typically move about on land for several days before entering aquatic habitats. M. Jones (personal communication) noted that hatchlings emerging from nests in August or September may move to wetlands, occupying forms usually near the margins, but whether they overwinter there or elsewhere is unknown.

Nevertheless, not all such wanderers reach water before cold weather arrives, and overwintering on land may not always be lethal. In Michigan, the occasional hatchling was caught at drift fences in spring (Congdon et al., ’83b, 2000), suggesting that they had successfully hibernated terrestrially. In Minnesota, 26 of 1,590 hatchlings collected at drift fences were captured in spring (Pappas et al., 2000). Because in both cases it is unlikely that these hatchlings had found other water in which to hibernate, it appears that they successfully overwintered on land, either within the natal nest or elsewhere. Terrestrial hibernation would be aided by this species’ well-developed capacity for freeze tolerance (Packard et al., 2000; Dinkelacker et al., 2004; Costanzo et al., 2006), which, in turn, is promoted by its high susceptibility to inoculative freezing (Packard et al., 2000; Dinkelacker et al., 2004). Thus, the available information suggests that autumnal emergence is normally followed by movement (albeit not necessarily direct) to water for overwintering, that some hatchlings may be stranded on land during the winter, and some unknown percentage of the latter can survive until the following spring. Given the mild temperatures they may encounter under snowpack, overwintering in terrestrial sites cannot be ruled out (Congdon et al., 2000).

_C. guttata_ (spotted turtle; Emydidae): Most studies that mention hatchlings of this species concern northern populations, although _C. guttata_ ranges southward to Florida. Numerous reports suggest that some hatchlings may emerge from nests in spring (Conant, ’38; Nemuras, ’67; Ernst, ’75, ’76; Chippindale, ’89). This notion was based on findings of turtles that were still hatchling-sized in spring and presumably had just emerged from their nests. However, an alternative explanation is that these hatchlings actually emerged in autumn and overwintered elsewhere, but did not grow before spring. Investigators who have directly monitored nests and/or paid special attention to hatchlings all report autumnal emergence (Belmore, ’80; Joyal, ’99; Carroll and Ultsch, 2007). Nevertheless, there are enough anecdotes of hatchlings captured abroad in spring that at least occasional terrestrial overwintering cannot be ruled out; further studies with monitored nests are required to make a definitive statement.

_Clemmys_ [Actinemys] marmorata_ (western pond turtle; Emydidae): This species ranges along the west coast of the United States and Mexico, from Baja western California into Oregon and parts of Washington (Ernst et al., ’94). Across its

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2See Footnote 1.
range, winters vary from mild to cold, although the hatchlings commonly hibernate inside the natal nest (Buskirk, '91; Reese and Welsh, '97; Rathbun et al., 2002; Van Leuven et al., 2004). As with most species whose hatchlings overwinter in the nest, autumnal emergence does occur occasionally, even as far north as Washington (F. Slavens, K. Slavens, and D. Anderson, personal communications), although it is more common in the southern portion of the range, where hatchlings may emerge in autumn or spring (Holland, '94; Hays et al., '99; Bettelheim, 2004). Although adults reportedly may overwinter on land or underwater (Holland, '94), hibernacula used by adults wintering by hatchlings, if it occurs, is unknown.

*C. picta* (painted turtle; Emydidae): There are four recognized subspecies of painted turtles, three of which occur as far north as Canada: C. *p. picta* (eastern painted turtle) in the eastern seaboard states and provinces; C. *p. marginata* (midland painted turtle) in the Great Lakes area; and C. *p. bellii* (western painted turtle) from the Great Plains westward to the Pacific Ocean. The fourth subspecies, C. *p. dorsalis* (bog turtle), is a form of the lower Mississippi River drainage and ranges from southern Missouri to the Gulf Coast. It had been long suspected that overwintering in the nest may occur in the northern subspecies (C. *p. bellii*—Thacker, '24; Woolverton, '63), based largely on observations of what appeared to be numerous newly hatched turtles abroad in spring, and/or the lack of similar observations in autumn. Many later studies, in which investigators caged nests to determine precise emergence dates or used drift fences to capture hatchlings as they migrated to water, have verified that the majority of the northern hatchlings spend their first winter inside the nest cavity (C. *p. bellii*—Christiansen and Gallaway, '84; St. Clair and Gregory, '90; Lindeman, '91; Costanzo et al., '95b, 2004; Pappas et al., 2000; C. *p. marginata*—Tinkle et al., '81; Costanzo et al., 2004; C. *p. picta*—Finneran, '48; Ernst, '71; DePari, '96; Rozycki, '98; Carroll and Utsch, 2007). There have not been as many reports on C. *p. dorsalis*, but overwintering in the nest occurs frequently as far south as Louisiana and Mississippi (Cahn, '37; Cagle, '54).

*Trachemys* spp. (sliders; Emydidae): *Trachemys scripta elegans*, the red-eared slider, is the only member of this group with a significant northern element to its range, which extends from the Gulf coast to northern Illinois and Indiana. In Illinois, emergence from the nest occurs almost entirely in spring (Cagle, '44; Smith, '61; Tucker, '97, '99, 2000a; Willette et al., 2005). Overwintering in the

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3See Footnote 1.
4See Footnote 1.
nest also occurs in northern Indiana (Costanzo et al., 2001c; P. Baker, J. Iverson, P. Meyer, personal communications) and possibly in Iowa (Christiansen and Vandewalle, 2000). Hatchlings appear not to be particularly freeze-tolerant (Churchill and Storey, ’92b; Packard et al., ’97c; Dinkelacker et al., 2005b; Costanzo et al., 2006). They also do not supercool appreciably when in contact with frozen soil, evidently because the skin offers little resistance to the penetration of ice crystals (Packard et al., ’97c; Costanzo et al., 2001b). Hence, overwintering in the nest requires that the soil about the hatchlings does not freeze, which in turn may limit the northern distribution of the species, given that the hatchlings do not opt to overwinter aquatically.

The hibernation habits of slider hatchlings are not so clear in the southern portions of the range. Cagle (’44) cited what he considered a reliable source, who said that emergence of red-eared sliders in Tennessee was approximately evenly divided between fall and spring. One turtle farmer in Louisiana (J. Evans) told us that T. s. elegans emerge in both autumn and spring on his farm, but that in the field, hatchlings apparently emerge mostly in spring. Another (H. Kleibert) said that any eggs not harvested in autumn produce hatchlings that emerge the following spring, and overwintering of hatchlings inside the nest is also the case in the field. In contrast, Cagle (’50) found that 34 natural nests in Louisiana “hatched” in July–September; however, one cannot assume that this means that the hatchlings emerged, as this was not specifically stated, especially as he also stated that they often overwinter in the nest, and that a farmer in the New Orleans area found many hatchlings in his field each spring. The appearance of hatchlings abroad in spring has been noted elsewhere (T. Mann [Mississippi], D. Nelson [Alabama], D. Haynes, F. Rose [Texas], personal communications); therefore, it appears that overwintering in the nest occurs throughout the range, with possibly a trend toward increasing, but still minor, fall emergence at lower latitudes.

Trachemys s. scripta: Trachemys s. scripta, the yellow-bellied slider, is a southeastern subspecies. In South Carolina, hatchlings typically hibernate inside the natal nest, although some fall emergence occurs. In Virginia, at least some hatchlings leave the nest in autumn and hibernate elsewhere (Mitchell, ’94). In northern Florida, 13 of 15 hatchlings found crossing roads were encountered in March and April (Jackson, ’94), although in peninsular Florida, nest emergence usually occurs before winter (Jackson, ’88). Aresco (2004), who excavated and reburied eggs in northern Florida, found that hatchlings emerged in both autumn and spring, although relative numbers were not reported. In New Mexico, a nest of the Big Bend slider (T. gaigeae) contained hatchlings in January that were gone in April, suggesting that the turtles had overwintered successfully (Morjan and Stuart, 2001). In summary, it appears that the majority of sliders overwinter inside the natal nest, although some fall emergence occurs in the more southern portions of their range.

Pseudemys spp. (red-bellied turtles and cooters; Emysidae): The Florida cooter (P. floridana floridana6) occurs on the coastal plain from Virginia to Alabama, exclusive of peninsular Florida, which is occupied by the peninsular cooter (P. f. peninsularis7). Gibbons and Nelson (’78) trapped all but one of 32 hatchling P. f. floridana during spring at a drift fence in South Carolina. During 1968–1998, at a drift fence encircling a South Carolina bay, 84.8% of 119 hatchlings were captured in March and April and only two were captured in September–November (J. Gibbons and J. Greene, unpublished data; see also Gibbons and Coker, ’77). In Alabama and the Florida panhandle (Walton County), most of the hatchlings collected by Thomas (’72) were taken in spring, leading to the presumption that hatchling P. f. floridana overwinter in the nest. In north Florida, Jackson (’94) observed four hatchlings crossing roads in spring, but found none in autumn, and Aresco (2004) reported that hatchlings emerged from artificial nests in both autumn and spring. In Alabama, some hatchlings emerge in fall, although the majority hibernate inside the nest (Thomas, ’72).

In peninsular Florida, P. f. peninsularis nests from fall through spring and is considered a “winter nester,” as is the chicken turtle (Deirochelys; see below) in this area (Ewert, ’91). Hatchlings have been found abroad in autumn

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6Considered a subspecies of Pseudemys concinna by the Committee on Standard English and Scientific Names (Iverson et al., 2008).
7Considered a full species, Pseudemys peninsularis, by the Committee on Standard English and Scientific Names (Iverson et al., 2008).
(Iverson, ’77; Jackson, ’88), although there are no data regarding when the majority of nest emergence occurs.

In Illinois, P. concinna (river cooter) hatchlings are found abroad in autumn (Cahn, ’37), whereas in northern Florida, of the 11 nests studied by Jackson (’94), hatchlings emerged from six in autumn and five in spring; there was no clear tendency for hatchlings in late-constructed nests to hibernate in situ. In Alabama, hatchlings sometimes overwinter outside the nest, but the tendency is to remain inside (Fahey, ’87). In West Virginia, Buhlmann and Vaughn (’91) collected five P. concinna they considered to be hatchlings in April–June, which they presumed had overwintered in the nest; however, at 32–42 mm, these individuals were large as compared with the 27–36 mm size given for hatchlings by Ernst et al. (’94); therefore, more definitive evidence of overwintering would be needed to affirm their supposition.

D. Nelson ([Alabama] personal communication) had found hatchling P. alabamensis (Alabama red-bellied turtle) during spring roadkill surveys, suggesting that these turtles hibernate inside the natal nest. In Florida, the Florida red-bellied turtle (P. nelsoni) appears to emerge in fall in the northern part of the state (Lardie, ’73; Jackson, ’88), but the situation in the southern part is unclear (Lardie, ’73); in any event, data are scarce for this species.

Mitchell (’74, 2003) reportedly observed red-bellied turtle (P. rubriventris) hatchlings emerging from nests in April and in September in Virginia, but farther north, T. Graham (personal communication) has collected many hatchlings abroad, all in autumn, from an isolated population in Massachusetts. This is a reversal of the situation in most other species of facultative terrestrial hibernators, wherein overwintering in the nest tends to be more common in the north than in the south. In Maryland, Swarth (2004) determined that emergence of P. rubriventris hatchlings from 17 nests was split about evenly between fall (n = 60) and spring (n = 85) and that timing was not correlated with oviposition date. For one female that laid three nests over 2 years, a nesting on 7 June produced hatchlings that emerged in spring; hatchlings from a 29 June nesting emerged in autumn and overwintered elsewhere; and a second nest laid on 12 July of the same year produced 12 hatchlings that remained in situ, but died during winter. These findings are bolstered by a second study at the same site (Friebele and Swarth, 2005): of 31 monitored nests, 16 produced hatchlings that hibernated inside the nest, 10 produced hatchlings that emerged in autumn and overwintered elsewhere, and 5 had hatchlings that emerged in both autumn and spring. In total, 163 hatchlings hibernated inside the nest and 126 hibernated elsewhere, with some females producing hatchlings exhibiting either behavior. Again, there was no correlation between emergence timing and date of oviposition. Neither nest temperature nor rainfall influenced season of emergence, although in spring, rainfall and rising temperature did stimulate nestling emergence, as was found for red-eared sliders in Illinois (J. Tucker, personal communication). In summary, the timing of hatchling emergence of the complex of red-bellied turtles appears to be somewhat plastic, perhaps dependent on the micro-environment within a given nest or even among eggs within a single nest.

Graptemys spp. (map turtles; Emydidae): In general, map turtles are a southern group; however, several species range into northern areas, especially the common map turtle (G. geographica), which is found in southern Canada, the Great Lakes region, and northern Vermont. Overwintering in the nest in northern areas seems to be the more prevalent habit. In Minnesota, Pappas et al. (2000) captured 38 of 45 hatchlings moving from land to water at a drift fence in spring, and in Iowa, the only two hatchlings similarly caught at drift fences by Christiansen and Gallaway (’84) were captured in spring. By monitoring individual nests, Nagle et al. (2004) found that the incidence of hatchlings overwintering inside the natal nest was 95% (n = 75 nests); Baker et al. (2003) found the same percentage (n = 20 nests) in a northern Indiana population. Overwintering inside the nest has been suspected by others, mostly on the basis of capturing hatchlings abroad in spring and finding them scarce in autumn (Newman, ’06; McCallum, 2003). However, some fall nest emergence occurs in northern areas, although it is usually minor (Newman, ’06). The only reports stating that overwintering inside the nest does not occur (or occurs rarely) in this species, as well as in G. ouachitensis and G. pseudogeographica at the same Wisconsin locale, are those of Vogt (’80, ’81), who did not directly monitor nests. Thus, most evidence indicates that hatchlings in northern populations remain inside nests during winter. Reports for southern populations are lacking, but, near Birmingham, AL, R. Guthrie (personal communication) observed hatchling G. geographica abroad in spring.

J. Exp. Zool.
The hibernation habits of hatchlings of other species of map turtles have been less well studied. Vogt ('80, '81) unequivocally stated that both G. ouachitensis and G. pseudogeographica in Wisconsin emerge from nests in autumn. In Indiana, M. Ewert (personal communication) found hatching G. ouachitensis abroad in spring, whereas, in Missouri, J. Tucker (personal communication) found them abroad in autumn. Similarly, Cahn ('37) found hatching G. pseudogeographica in autumn in Illinois. In contrast, in Minnesota, M. Pappas (personal communication) observed many hatchlings of this species migrating to water in spring, and a drift fence deployed in Iowa captured hatchlings in spring, but not in autumn (Christiansen and Gallaway, '84). In the southern United States, hatchlings of G. flavimaculata (Anderson, '58; Horne et al., 2003), G. nigrinoda (Lahanas, '82); G. gibbonsi (R. Jones [who monitored caged nests in Mississippi], personal communication), G. pulchra (Shealy, '76), G. barbouri (Wahlquist and Folkerts, '73), and G. oculifera (Anderson, '58) typically emerge from nests in autumn and presumably hibernate aquatically. The available information for Graptemys indicates that the more northerly species hibernate in the nest in both the northern and southern parts of their range, but principally southern species emerge from nests in autumn; thus, overwintering in the nest may be a fixed trait in some species that is independent of latitude. In cold climates, terrestrial hibernation is promoted by a well-developed capacity to supercool (Baker et al., 2003).

E. orbicularis (European pond turtle; Emydidae): We include this species because of its wide distribution in Europe and western Asia, the abundant information on its biology, and its proximate phylogenetic relationship to North American turtles of the genera Clemmys (including Actinemys and Glyptemys) and Emystoidae (formerly Emys). In Poland, clutches may fail to develop due to cold, may emerge from nests in autumn and hibernate elsewhere, may successfully overwinter (up to 93% survival) as hatchlings inside nests, or may burrow into the soil below the level of the nest (Mitrus and Zemanek, 2000, 2003; Mitrus, 2005). Timing of nest emergence can vary considerably from year to year, apparently depending on the average summer temperature. Perhaps this dependence explains why hatchlings in other locales emerge from nests in both autumn and spring. For example, in Ukraine, overwintering reportedly occurs inside the nest (Szcerbak, '98) and outside the nest (Kuzmin, 2002; Maza-
(A. Widrig and R. Burke, personal communication). Most other reports have also indicated that fall emergence predominates (Burger, '76a, '77; Auger and Giovannone, '79; Mitchell, '94; Butler, 2004; Draud et al., 2004); however, it is possible that hibernation inside nests has gone unnoticed at some sites (Roosenburg, 2003). For example, in a 3-yr study in New Jersey, Baker et al. (2006) found that a significant number of hatchlings hibernated inside the nest. Overwintering success was ascribed to a well-developed freeze tolerance (see also Dinkelacker et al., 2005b; Costanzo et al., 2006), which is accompanied by a high susceptibility to inoculation from ice and ice-nucleating agents (INA) in the environment.

It is commonly assumed that turtles emerging from the natal nest in autumn ultimately overwinter in water. However, hatching *M. terrapin* often do not move directly to water (W. Roosenburg, personal communication), preferring to hide in the tidal wrack (Pitler, '85). Juveniles are found under vegetation up to 100 m from water (Pitler, '85). Terrestrial overwintering of hatchlings outside of the nest also occurs on Long Island, NY (Draud et al., 2004). Whether hibernation under water during the first winter is also common remains to be determined.

*Apalone* spp. (softshell turtles; Trionychidae): Both the smooth softshell (*A. mutica*) and spiny softshell (*A. spinifera*) range from the Gulf coast to the northern states and/or Canada, and therefore are found in areas with cold winters. Throughout their ranges, both species are known to emerge from their nests in autumn, presumably to allow aquatic overwintering (*A. mutica*—Muller, '21; Anderson, '58; Fitch and Plummer, '75; Plummer, '76, '77; Christiansen and Gallaway, '84; *A. spinifera*—Surface, 1908; Cahn, '37; Breckenridge, '60; Smith, '61; Christiansen and Gallaway, '84; Costanzo et al., '95b). Hatchlings are not freeze-tolerant (Costanzo et al., '95b) and owing to the large amount of exposed skin, they presumably would be highly susceptible to inoculation by ice and INA in the nest environment (Costanzo et al., 2001b). Softshells are also found in cold climates in Russia (e.g., *Pelodiscus sinensis*), where they, too, overwinter outside the nest (Kuzmin, 2002), presumably in an aquatic habitat. Limited cold hardiness could explain why hatching softshells leave the nest in autumn at high latitudes, but cannot account for similar behavior in more tropical populations. Because *A. spinifera* and *A. mutica* typically nest on sandbars, fall emergence may be a way to escape dehydration and winter flooding.

**Winter environment**

The physical environment to which hatching turtles are exposed in winter impacts their physiological condition and prospects for survival, and even their fitness after emergence from hibernation. Natural hibernacula are notoriously difficult to locate and study, especially during inclement conditions; therefore, there is a paucity of even fundamental information about them. This is unfortunate because their physical characteristics govern the exchange of heat, water, and gases by overwintering turtles. In turn, these factors drive evolution of strategies for coping with cold, dehydration, and hypoxia, the primary environmental challenges confronting hatchlings in winter.

Intolerance of environmental extremes may cause significant attrition of hatching turtles. Reports of widespread mortality among hatching turtles are not uncommon (Andreas and Paul, '98; St. Clair and Gregory, '90; Lindeman, '91; Packard, '97; Packard et al., '97a; Nagle et al., 2000) and demographic studies have shown that winter-kill may contribute significantly to mortality of this age class (Tinkle et al., '81), but few studies have identified the specific mortality factors. Hypothermia may be such a factor, especially in northern populations of terrestrial hibernators (St. Clair and Gregory, '90), as losses often are greatest in years of low temperatures and scarce snowfall (Breitenbach et al., '84; Andreas and Paul, '98; Schneeweiss et al., '98; DePari, '96; Nagle et al., 2000; Carroll and Ultsch, 2007). Terrestrial hibernators also succumb to flooding (Christens and Bider, '87; Holte, '88; DePari, '96; Rozycki, '98). Dehydration may contribute to winter mortality, either directly or indirectly (Christiansen and Gallaway, '84; Schneeweiss et al., '98; Packard et al., '97a; Costanzo et al., 2001b). Environmental stressors may interact in ways that have particularly significant effects on overwintering success. For example, cold and wet winters are particularly challenging to hatching *C. picta* hibernating within natal nests (Rozycki, '98; Costanzo et al., 2004), probably owing to increased risk of freezing mortality. Below, we summarize the available information concerning physical features of turtle hibernacula.

**Thermal regime**

Hatching turtles are small, ectothermic organisms whose heat content closely tracks changes in ambient temperature. Nevertheless, there is some evidence for metabolic thermogenesis in turtle
nests (Bustard, '71; Burger, '76b). During emergence from their nest, loggerhead turtles (Caretta caretta) reportedly liberate enough heat that the surrounding sand warms noticeably (Moran et al., '99), but it is unclear whether this heat was endogenously produced or whether the turtles simply radiated heat they had gained from deeper, warmer places in the soil column. Even hibernating hatchlings release small amounts of metabolic heat, but there is no evidence that this heat significantly alters their thermal environment. Hatchlings liberate a substantial amount of heat (335 J g⁻¹) when water in their tissues freezes, but whether or not this heat could impact the thermal dynamics of other turtles sharing the hibernaculum has not been determined.

Investigators have attempted to characterize the thermal conditions affecting hatchling turtles in natural hibernaculum (Breitenbach et al., '84; DePari, '88; Schneeweiss et al., '98; Packard et al., '89, '97a; St. Clair and Gregory, '90; Costanzo et al., '95b, 2004; Packard, '97; Weisrock and Janzen, '99; Nagle et al., 2000). This task is simplified by deploying miniaturized temperature recorders directly inside occupied turtle nests. To avoid disturbance of the nest chamber, investigators sometimes place these units adjacent at an appropriate depth in the soil column; resulting data satisfactorily represent nest temperatures (Breitenbach et al., '84; Nagle et al., 2000; Costanzo et al., 2004).

Recordings of temperatures within northern natal nest hibernacula commonly show that average daily temperature gradually decreases from ~15°C in October to near 4°C in December and slightly lags the seasonal decrease in air temperature (see Costanzo et al., '95b, 2004). Warming of the nest chamber, following a reversal of this pattern in spring, usually begins in early March. Examined on a finer scale, temperature recordings often show daily fluctuations of up to several degrees Celsius. In winter, such oscillations, which correspond to diel heating and cooling cycles, may expose hatchlings to frost on a nightly basis. During the coldest segment of winter, typically from late November through early March, temperatures inside nests may or may not descend appreciably below the equilibrium freezing point of turtle tissues, ~0.6°C. Despite the popular notion that hatchlings encounter a winter-long period of intense cold, data suggest that frost exposure is transient, even in severe winters (Table 1).

Investigators have lavished attention on the severity of the thermal conditions within individual hibernacula, particularly with the aim of linking them to observations of hatching mortality (e.g., Weisrock and Janzen, '99). Drawing inferences about this association hinges on the assumption (probably true) that freezing is a major cause of winter mortality; factors influencing freezing risk, such as seasonal minimum temperature and frost duration, are the most critical elements of such analyses (for a discussion, see Lee and Costanzo, '98). Because freezing risk can fluctuate with changes in certain environmental variables, frequency of frost exposure is also an important metric.

Some analyses (Costanzo et al., '95b, 2004; Packard, '97; Packard et al., '97a; Baker et al., 2003, 2006) of the thermal conditions inside terrestrial hibernacula have enumerated and characterized individual bouts of frost exposure, each representing a discrete period of chilling at temperatures that are at or below the equilibrium freezing/melting point of turtle tissues, ~0.6°C. One assumption implicit in these analyses is that the risk of freezing (and death) increases with the frequency and duration of such exposures, and also with the severity of the cold. The consensus of these studies is that the typical subzero chilling excursion is relatively benign, as turtles commonly encounter temperatures only slightly below ~0.6°C. An analysis of the thermal relations of hatchling C. picta in the Nebraska Sandhills, for example, showed that hibernaculum (natal nest) temperature was within 2°C of the tissue freezing point for 30–50% of the total period of freezing risk (Costanzo et al., '95b). During an exceptionally cold winter, the typical chilling excursion exposed hatchlings to temperatures above ~3.5°C and lasted only ~24 h (Table 1). The analysis also showed that, although hatchlings in deeper nests would have encountered fewer frost exposures, varying the nest depth from 5 to 25 cm had little effect on minimum temperature, duration, or maximum cooling rate.

There is substantial geographic variation in thermal dynamics and severity of cold inside hibernacula used by hatchling turtles. In regions where snowfall is abundant, temperatures in natal nests often hover around the freezing mark and instances in which hatchlings actually encounter frost are relatively uncommon (Obbard and Brooks, '81a; Breitenbach et al., '84; Schneeweiss et al., '98; Paulkstis et al., '89; Rozyczki, '98; Nagle et al., 2000; Costanzo et al., 2004). During periods...
of severe weather, nest temperature may dip slightly below freezing, but an insulating snowpack usually renders this peril modest and brief (i.e., hours to days). However, hatchlings may encounter extreme cold on the rare occasion that snow is lacking (e.g., Rozycki, ’98; Weisrock and Janzen, ’99; Nagle et al., 2000). In Acadia National Park, ME, temperature in three C. picta nests vacillated between 0 and −8°C, reaching minima of −10.7 to −12.6°C during January and February owing to a lack of snow cover. Snow fell in March and nest temperatures moderated, remaining near 0°C (Rozycki, ’98).

Thermal conditions differ considerably in locales where winters are extreme and snow cover is light and transient. One such place is the Sandhills region of central Nebraska. There, most chilling bouts in C. picta nests are brief, such that hatchlings cool only a few degrees below 0°C before rewarming; however, extended chilling can occur and turtles can reach critically low temperatures, sometimes, though not always, with lethal consequences (Costanzo et al., ’95b, 2004; Packard, ’97). Extreme chilling excursions occur infrequently and tend to be relatively brief, although they can profoundly impact cohort survival.

Several studies have demonstrated pronounced variability in the intensity and duration of subfreezing cold among hibernacula at the same locale (Breitenbach et al., ’84; Packard et al., ’89, ’97a; Paukstis et al., ’89; Packard, ’97; Weisrock and Janzen, ’99; Nagle et al., 2000). For example, seasonal minimum temperatures recorded in different C. picta nests vary considerably, ranging 10°C or more (Table 2). Such variation apparently reflects differences in nest depth, as well as slope, aspect, patchiness of snow cover, and other physiognomic factors (Weisrock and Janzen, ’99; Costanzo et al., 2004). Female C. picta choose nest sites that promote development of viable offspring and potentially influence their sex (Schwarzkopf and Brooks, ’87; Ewert et al., ’94; Janzen, ’94; Morjan, 2003), but whether or not they also select sites that minimize environmental stress on overwintering hatchlings is not clear (Weisrock and Janzen, ’99).

Developing embryos experience a substantial range in temperatures due to vertical thermal gradients within the soil column (Thompson, ’88; Georges, ’92; Tucker, ’99). By the same token, thermal conditions can also vary considerably within a single hibernaculum as, for example, hatchlings overwintering near the top of a nest could become colder than those remaining near the bottom (Costanzo et al., ’95b; Tucker, ’99). Thermal variation probably is stronger in shallow nests (e.g., Houghton and Hays, 2001) and in larger nests (i.e., those containing many hatchlings), and in soils prone to developing a sharp depth–temperature gradient, and potentially could explain the mixed survival occurring in some nests (MacCulloch and Secoy, ’83; DePari, ’88; Packard et al., ’89; Lindeman, ’91; Costanzo et al., 2004).

### Hydric regime

Water is critical to life and, if not obtained through feeding (likely the case in overwintering hatchlings), must be obtained from the environment, if possible, or by catabolism of endogenous fuel reserves. Water availability is obviously not an issue for hatchlings overwintering aquatically, but may be a significant problem for terrestrial hibernators. Indeed, desiccation and associated osmo-ionic perturbations may be a more formidable mortality factor than starvation in dormancy (Seidel, ’78; Gregory, ’82). On the other hand, excessive moisture and inundation can cut off

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**TABLE 1. Characterization of potential freezing episodes at a site in Garden County, NE, where painted turtles (Chrysemys picta bellii) overwinter inside nests**

<table>
<thead>
<tr>
<th></th>
<th>Minimum temperature (°C)</th>
<th>Duration (h)</th>
<th>Maximum cooling rate (°C h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical winter</td>
<td>Median (range)</td>
<td>Median (range)</td>
<td>Median (range)</td>
</tr>
<tr>
<td>16</td>
<td>−1.6 (−9.6 to −0.8)</td>
<td>8 (2–86)</td>
<td>0.33 (0.05–0.85)</td>
</tr>
<tr>
<td>Cold winter</td>
<td>−3.4 (−11.8 to −0.8)</td>
<td>23 (6–320)</td>
<td>0.58 (0.15–0.55)</td>
</tr>
</tbody>
</table>

Characteristics of potential freezing episodes (N), each a discrete sequence of soil temperatures below −0.6°C, the approximate equilibrium freezing/melting point of turtle tissues, during two winters of record. Temperatures were measured 10 cm below the surface of a sandhill and are representative of conditions inside nests containing hatchling C. picta. Cooling rate was determined from successive temperature recordings made at 2-h intervals. In the “typical” winter (1993–1994), the average daily air temperature for December and January was similar to the mean of these values (−3.8 and −4.0°C, respectively) over the preceding 15 yr; in the “cold” winter (1990–1991), the average daily air temperature for December and January was lower than the respective long-term means by 3.8 and 2.2°C, respectively. Adapted from Costanzo et al. (’95b).
oxygen supply and cause osmoregulatory perturbations.

There is little information about the hydric relations of neonates overwintering in natal nests or other terrestrial hibernacula. Hatchlings overwintering inside natal nests probably are exposed to a wide range of hydric conditions reflecting the diversity of oviposition sites; however, turtles generally nest in mesic sites because adequate moisture is required for proper development of incubating embryos (Packard and Packard, '88; Ratterman and Ackerman, '89; Cagle et al., '93). Hatchlings overwintering deep in the soil column, such as *K. flavescens* and *T. ornata*, probably encounter static, humid conditions. The few specimens examined were found above the water table (Costanzo et al., '95b; Converse et al., 2002), although in lowland situations inundation by rising ground water could occur. Inundation can also be problematic for hatchlings that hibernate inside natal nests. Riverine species, such as *G. geographica* and *G. pseudogeographica*, overwintering inside floodplain nests are susceptible to flooding, and hatchling *M. terrapin* hibernating on tidal flats may be inundated by storm surges (R. Wood, personal communication). Even species that commonly nest in upland sites are occasionally impacted by flooding or chronic soil saturation.

### Table 2. Survival and minimum temperatures to which hatchling turtles are exposed during hibernation inside natal nests

<table>
<thead>
<tr>
<th>Locale</th>
<th>Winter</th>
<th>Survival (%)</th>
<th>Minimum</th>
<th>Sample Period</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chrysemys picta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE British Columbia</td>
<td>1987–1988</td>
<td>3</td>
<td>0–10</td>
<td>–6 (1)</td>
<td>Jun–Mar</td>
</tr>
<tr>
<td>1988–1989</td>
<td>8</td>
<td>0</td>
<td>0.0</td>
<td>–5 (1)</td>
<td>Jun–Apr</td>
</tr>
<tr>
<td>St. Louis Co, MN</td>
<td>1961–1962</td>
<td>1</td>
<td>100</td>
<td>–11 (1)</td>
<td>Nov–Mayf</td>
</tr>
<tr>
<td>Cherry Co., NE</td>
<td>1987–1988</td>
<td>7</td>
<td>22–100</td>
<td>–6.2 to –0.2 (7)</td>
<td>Nov–Febf</td>
</tr>
<tr>
<td>1994–1995</td>
<td>14a</td>
<td>0–100</td>
<td>81.4</td>
<td>–11.3 to –2.9 (14)</td>
<td>Nov–Mar</td>
</tr>
<tr>
<td>1995–1996</td>
<td>18a</td>
<td>0–100</td>
<td>63.2</td>
<td>–21.0 to –3.0 (18)</td>
<td>Nov–Mar</td>
</tr>
<tr>
<td>Garden Co., NE</td>
<td>2000–2001</td>
<td>9</td>
<td>0–100</td>
<td>70.6</td>
<td>Jun–Mar</td>
</tr>
<tr>
<td>Carroll Co., IL</td>
<td>1995–1996</td>
<td>9</td>
<td>0–100</td>
<td>72.3</td>
<td>Nov–Mar</td>
</tr>
<tr>
<td>Morris Co., NJ</td>
<td>1985–1986</td>
<td>1</td>
<td>100</td>
<td>–10.2 (1)</td>
<td>Sep–Apr</td>
</tr>
<tr>
<td>1986–1987</td>
<td>1</td>
<td>100</td>
<td>–</td>
<td>–5.5 (1)</td>
<td>Jun–Decf</td>
</tr>
<tr>
<td>Central Ontario</td>
<td>1987–1988</td>
<td>3</td>
<td>100</td>
<td>100.0</td>
<td>Jun–Febf</td>
</tr>
<tr>
<td>Livingston Co., MI</td>
<td>1981–1982</td>
<td>9</td>
<td>100</td>
<td>100.0</td>
<td>Nov–Apr</td>
</tr>
<tr>
<td>1995–1996</td>
<td>8</td>
<td>n.r.</td>
<td>58.5</td>
<td>–7.5d</td>
<td>Nov–Mar</td>
</tr>
<tr>
<td>1996–1997</td>
<td>35</td>
<td>n.r.</td>
<td>98.9</td>
<td>–1.0d</td>
<td>Nov–Mar</td>
</tr>
<tr>
<td>1997–1998</td>
<td>16</td>
<td>n.r.</td>
<td>98.9</td>
<td>–4.4d</td>
<td>Nov–Mar</td>
</tr>
<tr>
<td>1998–1999</td>
<td>17</td>
<td>n.r.</td>
<td>100.0c</td>
<td>–2.4d</td>
<td>Nov–Mar</td>
</tr>
<tr>
<td><strong>Graptemys geographica</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fulton Co., IN</td>
<td>2000–2001</td>
<td>6</td>
<td>100</td>
<td>100.0</td>
<td>May–Jul</td>
</tr>
<tr>
<td>2001–2002</td>
<td>6</td>
<td>90–100</td>
<td>98.3</td>
<td>–5.4 to –3.6 (3)</td>
<td>May–Jul</td>
</tr>
<tr>
<td><strong>Malaclemys terrapin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cape May Co., NJ</td>
<td>2002–2003</td>
<td>6</td>
<td>0–100</td>
<td>80.4e</td>
<td>Jul–Mar</td>
</tr>
<tr>
<td>2003–2004</td>
<td>43</td>
<td>0–100</td>
<td>85.1e</td>
<td>–4.7 to –1.0 (8)</td>
<td>Jul–Mar</td>
</tr>
<tr>
<td><strong>Trachemys scripta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jersey Co., IL</td>
<td>1996–1997</td>
<td>7b</td>
<td>28–100</td>
<td>85.1</td>
<td>Jan–May</td>
</tr>
</tbody>
</table>

Survival represents the percentage of fully formed hatchlings in individual nests alive in spring; minimum nest temperature is lowest temperature observed inside or adjacent to individual nests (range is reported, n given in parentheses) during the indicated sample period. n.r., not reported. References: (1) St. Clair and Gregory ('90); (2) Woolverton ('83); (3) Packard et al. ('89); (4) Packard et al. ('97a); (5) Packard ('97); (6) Costanzo et al. (2004); (7) Costanzo et al. (2003); (8) Weisrock and Janzen ('99); (9) DePari ('88); (10) Storey et al. ('88); (11) Breitenbach et al. ('84); (12) Nagle et al. (2000); (13) Nagle et al. (2004); (14) Baker et al. (2003); (15) Baker et al. (2006); (16) Tucker and Packard ('98).

aNatural nests were stocked in late October with turtles hatched in the laboratory.
bArtificial nests were stocked in mid-October with turtles hatched in the laboratory.
cAverage for all hatchlings observed, not individual nests.
dTemperature of soil at the average depth of nest bottom, 7.5 cm.
eRecorded near the top of the nests.
fDiscontinuous sampling regimen probably underestimated true winter minimum temperature.
Dynamics of water exchange in terrestrial hibernacula can have potentially significant implications on the physiology and cold hardiness of overwintering hatchling turtles. Percolation of rainwater through the soil column may flush carbon dioxide from the nest chamber and deliver oxygen to hatchlings (Packard, '91). Moisture in soil also influences thermal conductivity and thus rates at which turtles heat and cool, and the frequency and duration of frost events. An abundance of environmental moisture can benefit hatchlings by reducing EWL. However, this condition also promotes inoculative freezing of turtles, an outcome that may be advantageous (Dinkelacker et al., 2004; Baker et al., 2006) or deleterious (Baker et al., 2003), depending on the species. By periodically sampling soil from C. picta nests, Costanzo et al. ('98, 2004) found that soil moisture levels may peak in mid-winter, when nest temperatures are lowest and the risk of freezing is therefore the greatest. These studies also showed that the moisture regime is linked to edaphics and regional precipitation patterns, but also varies markedly among hibernacula at a given nesting locale. Such variation may strongly impact turtle physiology, particularly with respect to water balance, and may also govern risk of freezing and, possibly, mortality (Costanzo et al., 2004).

Methods for sampling gas from inside turtle burrows (Ultsch and Anderson, '86) and intact experimental nests (Wallace et al., 2004), have been devised but whether or not gas exchange poses problems for terrestrially overwintering hatchlings has yet to be investigated. Ventilation of subterranean hibernacula occurs by diffusion of respiratory gases through spaces among soil particles and their ultimate exchange with the free atmosphere. Consequently, gas tensions are influenced by soil texture, porosity, particle size, water content, temperature, rainfall, and other factors affecting diffusion (Congdon and Gibbons, '90; Booth, '98; Shams et al., 2005). Gas tensions inside hibernacula are also influenced by respiration of the hatchlings as well as microbes and gases.
other resident organisms (Ackerman, ’77). Some research has shown that \( PO_2 \) and \( PCO_2 \) can vary among regions of a given nest as a function of the interplay between diffusion and metabolic activity of the occupants (Ralph et al., 2005).

Gas tensions inside nests containing developing embryos often approximate those in ambient air, although hypoxia and hypercapnia conceivably could develop under certain circumstances. For example, diffusion coefficients are especially low in soils rich in clay (Shams et al., 2005) and organic matter (van der Lee et al., ’99), probably because they have little air space and tend to retain water, which has a relatively low gas conductance. Snow deposition and freezing of the soil solution could hamper diffusion even in porous soils. Because ground water usually contains little dissolved oxygen (Malard and Hervant, ’99), environmental hypoxia can result from inundation caused by a rising water table (Christens and Bider, ’87; Kam, ’94). Thus, environmental constraints on gas exchange potentially could influence whether hatchlings of a given species can successfully hibernate in terrestrial situations.

**Ice-nucleating agents**

A host of terrestrial environments, including ones in which hatchling turtles overwinter, harbor natural substances that catalyze the freezing of supercooled water. These substances, commonly called INA, act by orienting water molecules in a geometric configuration favoring the formation of an ice embryo, which in turn precipitates ice crystal growth (Lusena, ’55; Langham and Mason, ’58; Vali, ’95). INA are important elements of the winter microenvironment because, once ingested or otherwise incorporated into an organism’s tissues, they sharply limit supercooling capacity (Lee and Costanzo, ’98; Zachariassen and Kristiansen, 2000). With hatchling turtles, contamination with INA can reduce supercooling capacity by 8–10°C (Costanzo et al., 2000a, 2003).

INA commonly found in soils include mineral particulates, such as quartz and silicates (Mason and Maybank, ’58; Roberts and Hallett, ’68; Kumai, ’76; Shen et al., ’77), organic crystalloids and organic/inorganic complexes formed during decay (Power and Power, ’62; Fukuta, ’66; Vali, ’91), and certain bacteria and fungi (Hirano and Upper, ’95; Upper and Vali, ’95). Although the soils in which deeply burrowing turtles hibernate have not been examined, it is clear that upper soil horizons generally support more kinds and greater concentrations of INA than do deeper strata (Vali, ’91) and therefore these agents probably are of greatest importance to hatchlings that overwinter within natal nests or other superficial hibernacula. Turtle nests harbor INA of at least two major classes: inorganic particulates, which are active in the range −8 to −6°C, and small (<10 nm), water-soluble, organic entities that can express activity at temperatures as high as −3°C (Costanzo et al., ’98, 2000a, 2001c, 2003, 2004). Although both types can significantly constrain supercooling capacity of hatchling turtles, work in this area has focused on the latter, which apparently have the greater impact on turtle cold hardiness.

Water-soluble INA found in turtle nesting substrata are sensitive to autoclaving, heating, and exposure to pH extremes and thus are probably of organic, and possibly biogenic, origin (Costanzo et al., 2000a). Some evidence suggests that they are derived from or associated with ice-nucleating microbes. Samples of soil collected from inside *C. picta* nests exhibited an increase in ice-nucleating activity when incubated in the cold, and a subsequent loss of activity on rewarming (Costanzo et al., 2000a), possibly reflecting a thermally sensitive expression of genes encoding ice-nucleating proteins in microbes (e.g., Rogers et al., ’87; Gurian-Sherman and Lindow, ’95). Preliminary studies of microbial colonies cultured from these samples produced DNA sequences that matched primers for ice-nucleating proteins synthesized by ice-nucleating bacteria. However, microscopic examination of extracts prepared from these samples yielded no recognizable cellular structures, suggesting that the proteins had been shed into the environment (e.g, Phelps et al., ’86).

INA are ubiquitous in terrestrial environments and have been found at turtle nesting locations throughout North America (Costanzo et al., 2000a, 2001c, 2004; Baker et al., 2006). INA activity tends to be greater in wetter soils (Costanzo et al., 2004), but apparently is not strongly associated with abundance of organic matter (Costanzo et al., 2000a). Activity levels in soil exhibit a seasonal pattern, such that maximal activity coincides with the coldest part of winter, presumably when their effects on turtle cold hardiness would be greatest (Costanzo et al., ’98, 2000a, 2004). Soil inside nest hibernacula can vary considerably with respect to INA abundance and/or potency, perhaps owing to differences in incident precipitation and relief (Costanzo et al., ’98, 2000a). In an extreme case, ice-nucleating activity in soil collected from four

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C. picta nests in Ontario ranged from −11.2 to −3.4°C (Costanzo et al., 2000a). The environment inside these hibernacula can exhibit INA activity that is high relative to that in adjacent soils, suggesting that INA can become concentrated inside turtle nests; however, this is not always the case (Costanzo et al., 2004).

Edaphics

Turtles nest in diverse substrata, both natural and anthropogenic (e.g., DePari, ’88; Rozycki, ’98). The influence of physical characteristics of soil on hatching success and hatchling phenotype has received extensive study (Packard et al., ’87; Ratterman and Ackerman, ’89; Congdon and Gibbons, ’90), but relatively little attention has been paid to their impacts on the physiology and survival of turtles overwintering in terrestrial situations. Attributes of soil, such as texture (i.e., fractional composition of sand, silt, and clay), friability, and organic matter content, can influence hatchlings through their variable effects on thermal, hydric, and gas regimes within hibernacula (Fig. 3). For example, although porous, well-drained soils accelerate EWL, heavier soils, such as clays and clay loams, retain moisture, and thus tend to heat and cool relatively slowly. Friable soils are prone to infiltrating the nest cavity (Congdon and Gibbons, ’90), placing hatchlings at an increased risk of freezing through INA contamination and inoculative freezing (see below). There is little evidence that certain soils are strictly avoided for overwintering. DePari (’96) reported that New Jersey C. picta hatching in sandy soil tended to emerge from their nests in fall, whereas turtles hatching in clayey soil overwintered inside their nests. His observation could suggest that the latter substrate was preferred, but not necessarily that the former is inhospitable; elsewhere, this species hibernates in sandy soils, often with good success (Christiansen and Gallaway, ’84; Costanzo et al., ’95b, 2004; Packard, ’97; Packard et al., ’97a).

Attributes of a particular soil, such as texture, porosity, and organic matter content, strongly influence the abundance and distribution of ice within the soil matrix, thereby affecting the risk of inoculative freezing (Costanzo et al., ’98). Inoculative freezing of hatchlings occurs readily in substrata, such as sands, which attenuate little moisture. Additionally, although porous soils may drain well, they also contain large voids that can fill with ice to which hatchlings are intimately exposed. As a consequence, susceptibility to inoculative freezing (and possibly death) is far greater for animals exposed to sandy soil as compared with clayey soil, all else being equal (Packard and Packard, ’97; Costanzo et al., ’98, 2001c). In principle, therefore, variation in soil attributes on even a microgeographic scale can markedly influence winter survival rates. Costanzo et al. (’98) noted in reports of overwinter mortality (Packard, ’97; Packard et al., ’97a) that viability was in fact lower for C. picta hibernating inside nests constructed in fine sand as compared with loamy sand. Direct tests of this association further bolster the conclusion that regional and local variation in soil characteristics can impact overwintering success in hatchling turtles (Costanzo et al., ’98, 2001c).

Spatial and temporal variability

Given that turtles are distributed over broad ecological and climatological regimes, environmental conditions in hibernacula must vary tremendously. In the north, ponds and marshes remain cold for extended periods with attendant implications for duration of dormancy in aquatic hibernators. In the same regions, the presence and persistence of ice cover, which strongly influences dynamics of gas exchange, bears on the physiological state of turtles. The microenvironment inside terrestrial refugia also varies with edaphics and a host of climatic factors, such as precipitation and temperature. In cold climates, conditions that elevate freezing risk are principle threats to winter survival, whereas in more temperate regions, conditions promoting dehydration and energy depletion are probably more critical.
Environmental conditions to which overwintering hatchlings are exposed can vary even among hibernacula at the same locale. For example, temperatures inside natal nests can be mild or severe depending on the depth and structure of the hibernaculum, and on local edaphics, topography, and physiognomy; such microgeographic heterogeneity can account for inter-clutch variability in the physiological state (Costanzo et al., 2004) as well as overwintering success (DePari, ’88; Packard et al., ’89, ’97a; Packard, ’97; Nagle et al., 2000; Costanzo et al., 2004). This scenario raises some interesting questions about the fitness implications of nest-site selection. Maternal choice of nesting site is an important, albeit little studied, factor shaping the life-history evolution of oviparous, precocial organisms (Resetarits, ’96). A female provides her offspring a measure of care through her choice of nesting site, which has portentous implications for embryo survival (Hughes and Brooks, 2006). Because the nesting landscape is a complex, temporal mosaic of varying physical conditions, choice of the oviposition site can strongly impact embryonic development and nest success (Congdon and Gibbons, ’90; Cagle et al., ’93; Kolbe and Janzen, 2001; Hughes and Brooks, 2006) as well as hatching phenotype (Schwarzkopf and Brooks, ’87; Packard and Packard, ’88). The consequence of nest microenvironment on hatching performance or fitness, including cold tolerance (Du et al., 2006), has received considerable study (Miller et al., ’87; O’Steen, ’98; Finkler, ’99; Rhen and Lang, ’99; Tucker and Paukstis, ’99; Finkler et al., 2000; Kolbe and Janzen, 2001; Steyermark and Spotila, 2001; Janzen and Morjan, 2002); however, the influence of nest-site location on winter survival of turtles has received scant attention (Breitenbach et al., ’84; Weisrock and Janzen, ’99; Nagle et al., 2000). The question of whether or not female turtles choose nest sites that are advantageous for hibernation within the nest remains open.

Environmental variability occurs on yet a finer scale: among regions of a given hibernaculum. Eggs occupying a three-dimensional nest chamber are exposed to a range of thermal (Ratterman and Ackerman, ’89; Tucker, ’99) and hydric (Legler, ’54; Hotaling et al., ’85; Marco and Díaz-Paniagua, 2008) conditions, such that position within a clutch may modulate expression of phenotypic traits that ultimately affect fitness (Packard and Packard, 2001b). For hatchlings that defer emergence from the nest until spring, such heterogeneity could explain instances of sibling variation in water balance, energy stores, cold hardiness, and ultimately winter survival (Costanzo et al., 2004).

Hatchlings overwintering within the nest typically form a cluster, their heads near the top of the nest chamber (Breitenbach et al., ’84; Andreas and Paul, ’98; Packard et al., ’89; St. Clair and Gregory, ’90; DePari, ’96; Tucker, ’97; Nagle et al., 2000). Fragments of eggshell on the chamber floor and walls may insulate turtles from contact with soil. However, if the clutch is relatively large, the hatchlings become distributed vertically and laterally around the nest center, and some workers (Costanzo et al., 2000a, 2001c, 2004; Nagle et al., 2000) have questioned whether turtles near the periphery of the nest may be predisposed to inoculative freezing via contact with ice crystals and INA. This scenario pertains only to nests that remain relatively free of infiltrating soil. However, as suggested by Costanzo et al. (2004), regional variation in soil texture and friability determines whether nests retain their form (Breitenbach et al., ’84; DePari, ’96) or become infiltrated (Hartweg, ’44; Packard et al., ’89). Nevertheless, differential freezing risk may explain the rather common instances of mixed survival of siblings overwintering in the same nest (Packard et al., ’89, ’97a; Lindeman, ’91; DePari, ’96; Weisrock and Janzen, ’99; Nagle et al., 2000; Costanzo et al., 2004; Carroll and Ultsch, 2007).

OVERWINTERING PHYSIOLOGY

The diversity of environmental conditions confronting hatchling turtles raises myriad physiological challenges to surviving winter. For aquatic hibernators, extreme cold and risk of freezing are not significant issues (Ultsch, ’89, 2006). On the other hand, prolonged submergence presents potentially severe problems of gas exchange, metabolic acidosis, and iono-osmoregulatory imbalance. Terrestrial hibernators must cope with dehydration and the attendant osmotic stress, and, if gas exchange is limited, hypoxia and hypercapnia. Hatchlings have no access to food and therefore must endure a prolonged period of aphagia. In northern regions, cold exposure presents a formidable challenge for the winter survival of hatchlings overwintering terrestrially.

Energetics

Generally, reptiles can endure remarkably long periods of aphagia (Frye, ’73). Hatchling turtles are provisioned with large stores of yolk that can
fuel metabolic activities at times when food is not available (Ewert, ’85; Congdon and Gibbons, ’90; Deeming, 2004b). This is particularly important for species whose hatchlings remain inside nests during winter or hibernate in the soil column, as feeding is not possible. Hatchlings emerging from their nests in autumn presumably have an opportunity to feed before winter, although the extent to which they do so remains uncertain. DePari (’88) found that aquatic invertebrate prey were as abundant during the period of autumnal emergence of hatchling C. picta as during the period of spring emergence. Hatchling G. insculpta have been observed feeding before hibernation (Tuttle and Carroll, 2005), but it is unknown how common this behavior is among other species. If feeding occurs, it will cease as the weather cools before hibernation; indeed, food intake is probably negligible at temperatures below 15°C (Cagle, ’50; Sexton, ’59; Ernst, ’71). Contrary to the experience of one of us (G. Ueltsch), neonatal C. serpentina did not eat when offered food in the laboratory (Finkler et al., 2002, 2004); some authors have surmised that these hatchlings defer feeding until the following spring (Sims et al., 2001). Thus, as both embryo and neonate, turtles may rely on energy provisioned by the mother for an extended period (Gibbons and Nelson, ’78).

Is energy limiting in the winter survival of hatchling turtles? Neonates usually have abundant energy reserves, primarily in the form of yolk lipids and proteins (Wilhoff, ’86; Congdon and Gibbons, ’90; Janzen et al., ’90). The quantity and quality of residual yolk is determined by the amount of nutrients maternally invested in each particular egg, as well as the thermal and hydric conditions prevailing during egg incubation (Deeming, 2004b). More energy is allocated to eggs in a female’s first clutch than in her second (Harms et al., 2005), but whether hatchlings resulting from the latter are disadvantaged for winter survival has not been studied. Residual yolk is a critical fuel for post-hatching activities, such as nest emergence and migration to other habitats. It is also critical to meeting basal metabolic needs during hibernation because most species probably are unable to (or simply do not) feed before winter. If the yolk supply is ample, some may be used for somatic growth, and body mass will increase during winter (Tucker et al., ’98; Filoramo and Janzen, ’99). However, if the supply is inadequate, other endogenous energy reserves are catabolized and the hatchling will tend to lose body mass (Finkler et al., 2002).

Ecological considerations

In the north, hatchlings must subsist on endogenous energy stores for extended periods. Fortunately, their hibernacula usually are quite cold and the depressive effect on metabolism can, to a large extent, offset the effects of prolonged aphagia. In milder climes, turtles probably go without feeding for shorter periods; however, owing to higher metabolic rates, it is unclear whether they fare any better energetically. This interesting conundrum has been considered for other ectotherms that also exhibit a wide geographical range (Costanzo, ’88; Irwin and Lee, 2000), but has not been resolved for hatchling turtles. It seems clear, however, that energy use is closely linked to prevailing ambient temperature. In a 2-yr study of hatchling C. picta in Indiana and Nebraska, Costanzo et al. (2004) found that utilization of stored lipids and other energy-yielding substrates was greater in the warmer winter, apparently reflecting a higher metabolic demand. In the same study, more energy was expended by hatchlings of the more temperate Indiana population, probably because winter temperatures were milder. In another study, hatchling T. scripta had less residual yolk following a mild winter than after a colder winter, this result portending the ill effects of global warming (Willette et al., 2005). Intuitively, it seems advantageous for turtles inhabiting relatively warm climes to provision their eggs with more nutrients in order that a large surplus be available for use after hatching, assuming that feeding before winter is not possible. Finkler et al. (2004) reported that solid matter, a proxy for energetic reserves, in eggs of C. serpentina exhibits a climatological cline, with populations experiencing higher temperatures before hibernation provisioning eggs with more solids. Energy provisioning of hatchlings can vary markedly among conspecific populations (Costanzo et al., 2004; Harms et al., 2005), perhaps because energy demand varies with local environmental conditions. An alternative strategy of energy conservation in relatively warm climes, which has yet to be explored in hatchling turtles, is to invoke behavioral and physiological mechanisms for reducing metabolic demand (Ewert, ’91).

Another intriguing, albeit unresolved, question pertains to the energetic consequences of hibernating on land vs. under water. Terrestrial hibernators presumably are exposed to relatively low prevailing temperatures, and, if gas exchange
is limited, low ambient gas tension could enhance the hypometabolic effect of cold (Burggren and Pinder, ’91). Still, avenues for rapid energy loss exist. In the event of tissue freezing, liver glycogen is rapidly converted to glucose (Churchill and Storey, ’91; Hemmings and Storey, 2000), which, if not reconverted after thawing, could be lost from the body via renal excretion (although we know little about the amount of renal excretion that occurs during winter).

Underwater hibernation presents a different suite of energetic considerations. In some amphibians and reptiles, apnea initiates responses that facilitate energy conservation, including bradycardia, peripheral vasoconstriction and oxygen rationing, and a decrease in metabolic rate (Shelton and Boutilier, ’82; Herbert and Jackson, ‘85; Donohoe and Boutilier, ’98; Ultsch et al., 2004). Hatchling turtles might drastically reduce energy use if they also exhibit the so-called “diving response,” although whether or not they do so is apparently unknown (Burggren and Pinder, ’91). Reese et al. (2004b) provided evidence that metabolism is reduced in hatchlings submerged in anoxic water as compared with hatchlings exposed to gaseous anoxia. In any case, a portion of the energetic savings probably is offset by the increased cost of managing salt and water balance necessitated by chronic submergence. Furthermore, submergence in hypoxic water would necessitate rapid depletion of glycogen reserves, particularly those of the liver, to mobilize glucose to the tissues (Warren et al., 2006).

Species whose hatchlings overwinter inside the nest reportedly (Congdon et al., ’83c; Congdon and Gibbons, ’85, ’90; Long, ’86; Rowe et al., ’95; Nagle et al., ’98; Costanzo et al., 2000b) have greater energy reserves than species whose hatchlings ultimately overwinter outside the nest (presumably aquatically), the favored interpretation being that terrestrial hibernation is energetically more costly. Unfortunately, there are no published comparisons of energy budgets for hatchlings hibernating terrestrially and aquatically. Preliminary work has shown that, during laboratory hibernation at 4°C, hatchling C. picta lost more yolk, dry liver mass, and dry carcass mass if submerged in normoxic water than if kept inside simulated nests (Fig. 4). The accelerated energy consumption in the aquatic hibernators, which remained quiescent throughout the experiment, probably resulted from efforts to maintain osmoregulatory balance, although water gain and reduced plasma osmolality suggested that such

![Fig. 4. Variation in energy consumption in hatchling painted turtles (Chrysemys picta bellii) during simulated hibernation on land and in water, as indicated by changes in dry mass of the carcass, liver, and residual yolk sac. Eggs were collected from gravid females at a nesting area in Garden Co., NE, and incubated in the laboratory at 29°C until hatching in August. Neonates (n = 24) were kept at 21°C on damp vermiculite until 1 October and then assigned to one of three groups. One group (initial) was immediately euthanized and assayed; another group (terrestrial) was placed in a simulated nest constructed of soil collected from the field site; another group (aquatic) was transferred to a plastic tank containing aerated water (8 cm deep) and rocks for shelter and basking. Aquatic turtles were first exposed to 15°C and indirect lighting (12L:12D) provided by a full-spectrum lamp; ambient conditions were changed to 10°C, 10L:14D on 1 November, and to 4°C, 10L:14D on 1 December. In mid-December, ice cover was simulated by reducing light intensity and installing a horizontal screen 3 cm below the water surface, thus preventing hatchlings from breathing air; these conditions were maintained thenceforth. Terrestrial turtles were exposed to the same thermal conditions, but kept in darkness. Hatchlings from both treatments were euthanized and assayed in mid-April. Means±SE (n = 8) identified with different letters were statistically distinguishable (ANOVA/Bonferroni; P<0.05). (J. Costanzo, B. Dishong, and T. Muir, unpublished data.)
efforts were incompletely successful. This experiment also showed that hatchling *C. picta* are capable of surviving winter in cold, normoxic water without access to air; in contrast, hatchlings of many (most?) species do not long tolerate anoxia (Reese et al., 2004b; Dinkelacker et al., 2005a,b).

**Physiological considerations**

Given the length of time during which hatchling turtles remain in negative energy balance, one might expect them to lose much body mass during winter. Such a loss could weaken the animal and adversely affect its fitness and survival. Field investigations suggest that success in migrating from the nest chamber to the relative safety of aquatic habitats is greater in heavier hatchlings (Janzen, '93; Tucker and Paukstis, '99). Changes in body mass of hibernating hatchlings obviously are linked to conditions prevailing during winter, although some evidence suggests that conditions during embryonic development are also important. For example, *T. scripta* developing in relatively dry nests tend to lose more mass in hibernation than turtles hatching in wetter nests (Tucker and Paukstis, '99).

Little is known about the dynamics of body mass during winter, except that limited information is available from laboratory studies. Only ~15% of body mass is lost during winter in hatching *G. geographica* (Nagle et al., 2004) and *T. scripta* (Congdon and Gibbons, '90). This amount generally concurs with the findings of Tucker and Paukstis ('99), who reported that *T. scripta* hatchlings lost 14–23% of their initial body mass during simulated terrestrial hibernation. Similarly, body mass was reduced 15–25% in hatchling *C. picta* kept under comparable conditions (Costanzo et al., 2003), but in neither study did the authors discover the extent to which the change reflected water loss, rather than nutrient consumption. In contrast to these findings, one field study, in which workers tracked the mean body mass of hatchling *C. picta* over a 6-month winter, showed that the decrease was slight, suggesting that energy use was minimal (Costanzo et al., 2004). Similarly, Baker et al. (2006) reported that the mean body mass of hatchling *M. terrapin* emerging from nests in spring was nearly identical to that of hatchlings emerging in autumn. In some cases, hatchling turtles overwintering in the laboratory lose weight sparingly, if at all (Tucker et al., '98; Filoramo and Janzen, '99; Costanzo et al., 2000b).

Much of the change in dry body mass undoubtedly reflects consumption of the residual yolk, the primary source of energy for overwintering hatchlings. In one laboratory study (Costanzo et al., 2003), yolk mass in *C. picta* on hatching decreased by 75–90% during a 5.5-month period of simulated hibernation. The remaining yolk weighed about the same as that measured in hatchlings collected in the field in spring; thus, energy use in the experimental animals probably was reflective of the natural situation. Similarly, little yolk remains in *T. scripta* on emergence from nest hibernacula in Jersey County, west-central Illinois (Tucker et al., '98). On the other hand, decrements in body mass and nutrient reserves can vary considerably among individual hibernacula and can be negligible at least occasionally (Costanzo et al., 2004).

Lipids are an important energy source in many hibernating reptiles (Derickson, '76; Gregory, '82). This may also be the case for the hatchlings of some turtle species (Wilhoft et al., '83), although caution is needed in making this generalization. During a relatively mild winter, hatchling *C. picta* catabolized only ~25% of their total lipid reserves and the resulting energy (~1.22 kJ) accounted for less than one-half of the total energy (2.70 kJ) produced from all substrates in the average-sized turtle (Costanzo et al., 2004). Furthermore, in the same investigation, turtles used lipids sparingly, if at all, during a subsequent, colder winter. This result could reflect severe hypometabolism and/or an inability to catabolize lipid at low temperature, particularly if cells become hypoxic during somatic freezing (Storey, '90b) and supercooling (Hartley et al., 2000; Costanzo et al., 2001a). Mechanisms of lipid sparing probably help to ensure the availability of a rich energy reserve to support post-emergence activities (Parker and Brown, '80; Gregory, '82; Costanzo, '85).

Other energy-yielding substrates, such as glycogen and proteins, apparently are important in the energy budget of overwintering hatchlings. Eggs of *C. picta* are provisioned with 2–3 times as much protein as lipid (Harms et al., 2005), and blood urea levels in hatchlings increase during fall and winter, suggesting that they actively catabolize proteins (Costanzo et al., 2004). Indeed, during simulated hibernation, hatchling *C. picta* (but not *C. serpentina*) maintain a rich pool of amino acids (Costanzo et al., 2000b), which can be metabolized even by hypoxic tissues (van Waarde, '88), the resulting carbon being converted to glucose via gluconeogenesis. This pathway may also be active.
during desiccation, as evidenced by the strong correlation between urea and glucose levels in some turtles (Costanzo et al., 2004). Amino acids can serve as “compatible osmolytes,” benign solutes that protect cells against dehydration, and some, such as proline, offer protection against both freezing and desiccation stresses (Takagi et al., 2000).

Turtles must limit energy expenditure during hibernation if they are to adequately fuel behavioral activities in spring. Little is known about mechanisms of metabolic regulation in hatchling turtles, although both physiologic and genetic factors are probably important. Resting metabolism in hatchlings of some species is influenced by environmental conditions prevailing during embryonic development (O’Steen, ’98; O’Steen and Janzen, ’99; Steyermark and Spotila, 2000). In northern regions, hatchlings undoubtedly benefit from extended periods of cold and its effect on biochemical processes; indeed, metabolism at subzero body temperatures is barely measurable (Halpern and Lowe, ’68; Voituron et al., 2002b). Additionally, in hatchling C. picta, chilling to near-freezing temperatures cause a marked slowing of energetically expensive functions, such as cardiac contraction (Birchard and Packard, ’97) and pulmonary ventilation (Larson, 2004), perhaps owing to an increased reliance on anaerobiosis or metabolic reprogramming. Even a modest decrease in ventilation frequency would be beneficial because, at least in older individuals, the majority of energy expended by inactive turtles is used for pulmonary respiration (Kinney and White, ’77).

Metabolic processes in overwintering hatchlings have not been studied in great detail. Larson (2004) investigated effects of thermal history and acute exposure to various temperatures on C. picta, using CO₂ release as a proxy for metabolic rate in a group of hatchlings that he successively acclimated to 15, 10, and 4°C. Metabolic rates at 10°C were essentially the same regardless of whether the hatchlings had been acclimated to 15 or 10°C. However, the rate measured at 4°C was significantly lower after turtles were acclimated to 4°C as compared with 15°C, suggesting that inverse compensation is a strategy of energy conservation in dormant hatchlings. Additional experimentation with 4°C-acclimated hatchlings elucidated the effect of varying temperature on metabolism. The mean rate of CO₂ release declined with temperature in an exponential manner, decreasing from 1.20 µLg⁻¹h⁻¹ at 15°C to 0.61 µLg⁻¹h⁻¹ at 10°C (Q₁₀ = 3.9), but only from 0.48 µLg⁻¹h⁻¹ at 4°C to 0.15 µLg⁻¹h⁻¹ at 0°C (Q₁₀ = 2.6). When exposed to subzero temperatures, hatchlings had extremely low levels of aerobic metabolism; for example, the rate of CO₂ release at −8°C was only 0.02 µLg⁻¹h⁻¹. This severe drop undoubtedly stems from the down-regulation of aerobic metabolic function, but may also reflect an increased reliance on anaerobic energy production with cooling into the subzero temperature range (see Voituron et al., 2002b).

The thermal sensitivity of metabolism in hatchling C. picta has also been investigated using closed-system respirometry (T. Muir, B. Dishong, and J. Costanzo, unpublished data). In this project, metabolism of hatchlings acclimated and tested at 15, 10, and 4°C varied exponentially with body temperature, although the Q₁₀ for this effect was considerably higher than that reported by Larson (2004); indeed, oxygen use at 4°C was only ~9% of that measured at 15°C (Fig. 5). A second aim of this study was to determine whether supercooling or freeze tolerance was the more energetically favorable strategy of cold tolerance in hatchling C. picta. For hatchlings exposed to −2.5°C, the mean rate of oxygen consumption was about three times greater in supercooled
(1.33 μL g\(^{-1}\) h\(^{-1}\)) vs. frozen (0.42 μL g\(^{-1}\) h\(^{-1}\)) animals. Interestingly, during recovery at 4°C, previously frozen C. picta consumed oxygen at a greater rate (3.50 μL g\(^{-1}\) h\(^{-1}\)) than turtles that had been supercooled (2.11 μL g\(^{-1}\) h\(^{-1}\)), perhaps because there is a latent energetic cost to freezing. In other freeze-tolerant vertebrates, repayment of an “oxygen debt” after thawing is sometimes observed (Layne, 2000), but may be absent, depending on severity of the freezing exposure (Voituron et al., 2002b). Hypometabolism at subzero temperatures could provide significant energetic savings. This is especially true of frozen animals in which the effect could result from enzymatic perturbation caused by the attendant rise in ion concentrations (Kanwisher, ’59). Moreover, a fall in tissue pH brought on by chilling, and perhaps accelerated by lactate accumulation, could create a “metabolic context” that favors development of metabolic depression (Storey and Storey, ’90).

**Respiration**

Exchange of respiratory gases is not problematic for hatchling turtles overwintering in ponds, streams, and other aquatic systems so long as they can breathe air. However, pulmonary ventilation could be difficult for cold, lethargic turtles, and, in temperate regions, is often prevented by ice formation. In these situations, diffusion across epithelia of the skin, buccopharynx, and cloaca becomes the only avenue for gas exchange (for a review, see Ultsch, ’89). Physiological mechanisms sustaining aerobic metabolism during submergence in oxygenated water include hypometabolism, a high efficacy of cutaneous gas exchange, and adaptations of the blood oxygen transport system (Ultsch and Jackson, ’82; Maginniss et al., ’83, 2004; Saunders and Patel, ’98). If the refugium becomes anoxic, which can occur in regions where ice cover persists, hatchling survival will then depend on a host of biochemical and physiological responses to low tissue PO\(_2\) (Jackson, 2002, 2004).

Whether or not gas exchange is problematic for hatchlings overwintering on land is unknown, although this scenario remains a possibility, particularly for animals hibernating deep in the soil column or in close proximity to other hatchlings, as is the case with species that hibernate inside their nest. Hypoxia and hypercapnia can develop in sea turtle nests when respiratory demands of late-stage embryos exceed the diffusive capacity of even highly porous soil (Ackerman, ’77; Ralph et al., 2005). Although there are far fewer propagules in the nests of freshwater turtles, these conditions might develop inside nests harboring overwintering hatchlings, especially if gas conductance is impeded by high clay content, water saturation, or freezing of the soil. Low temperatures in winter obviously help reduce oxygen demand and CO\(_2\) production; however, metabolism is relatively high in autumn and again in spring, just before emergence from hibernacula. Field studies are needed to determine whether or not gas exchange is a challenge to terrestrially hibernating hatchlings.

It is widely appreciated that, among vertebrates, anoxia tolerance is particularly well developed in turtles, although it certainly varies taxonomically, tending to be greater in northerly distributed forms that naturally encounter periods of hypoxia during long winters and persistent ice cover (Ultsch, ’89; Reese et al., 2004a; Ultsch and Reese, 2008). Research on this phenomenon has largely focused on survival and physiological responses in adult animals, which, except for terrestrial and estivating species, invariably hibernate aquatically. Physiological adaptations promoting survival during hypoxia include a depression of metabolism brought on by low ambient temperature and reduced oxygen availability; reliance on anaerobic pathways for energy production and tolerance of the associated lactacidosis; and an extensive mineral buffering system to manage acid–base disturbance (Lutz and Storey, ’97; Jackson, 2000a; Bickler and Buck, 2007). Anoxia-tolerant turtles also exhibit well-developed antioxidant defenses that minimize or prevent damage by reactive oxygen species (ROS) during reoxygenation (Willmore and Storey, ’97; Jackson, 2002; Milton et al., 2007).

Survival and physiological responses to anoxia in hatchling turtles have been examined only recently (Reese et al., 2004b; Dinkelacker et al., 2005a,b). The consensus of these studies is that anoxia tolerance varies taxonomically, as it does with adults, and is severely limited relative to that of conspecific adults. For instance, although adult C. picta can survive continuous submergence in cold, anoxic water for periods lasting 4–5 months (Ultsch and Jackson, ’82), survival of hatchling C. picta under similar conditions is limited to 40 d (Reese et al., 2004b). Survival in gaseous anoxia (N\(_2\) exposure) is even more restricted (Reese et al., 2004b), suggesting that submergence invokes responses that delay onset of physiological perturbation (although greater activity of hatchlings in
N$_2$ atmosphere is another possibility). Nevertheless, for the few species that have been examined, survival times in cold, anoxic submergence are only on the order of 1–6 weeks (Reese et al., 2004b; Dinkelacker et al., 2005a). The comparatively low tolerance relative to adults apparently reflects an incomplete ossification of the shell and an inadequate buffering system (Reese et al., 2004b; Dinkelacker et al., 2005a,b; Ultsch, 2006). Variation in shell composition and buffering capacity explains some of the taxonomic differences in anoxia tolerance among freshwater turtles (Jackson et al., 2007).

Hypercapnia, which potentially accompanies hypoxia, is a lesser-studied problem that could beset hatchlings crowded inside communal hibernacula with barriers to gas exchange. Lower vertebrates commonly respond to increased PCO$_2$ with a reduction in aerobic metabolism that may be precipitated by increased acidification (Storey and Storey, ’90). Hypercapnia stimulates ventilation in C. picta, although the effect is largely negated if the inspired gas is also hypoxic (Milsom and Chan, ’86). Adult aquatic turtles can become hypercapnic during the initial weeks of cold submergence, but this condition does not persist in the long term in either normoxic or anoxic water. If there is an acidosis associated with submergence, it is initially a combined respiratory and metabolic acidosis, but eventually becomes a pure metabolic acidosis, often partially offset by a slight respiratory alkalosis resulting from a fall in PCO$_2$ relative to air-breathing turtles (Jackson et al., 2000; Reese et al., 2001, 2002, 2003). Retention of CO$_2$ by hatchlings overwintering under water has not been investigated; however, given their small size and favorable geometry for cutaneous exchange, hypercapnia is unlikely to develop unless the PCO$_2$ of the surrounding water becomes elevated.

**Osmoregulation**

Water availability obviously is not a problem for turtles that hibernate in streams and ponds. Rather, excessive water influx is the primary osmoregulatory challenge confronting aquatic hibernators (Trobec and Stanley, ’71). Water deficit may pose serious difficulties for hatchlings overwintering in brackish water or on land. Terrestrial hibernators are subjected to dehydration when environmental water potential is low, such as when precipitation falls as snow rather than rain, or the soil solution freezes (Spaans and Baker, ’96). Because the vapor pressure of ice is lower than that of unfrozen body fluids, even supercooled organisms will lose water to a frozen environment (Forge and MacGuidwin, ’92; Lundheim and Zachariassen, ’93; Holmstrup et al., 2002). Behavioral, physiological, and morphological mechanisms of hydro-osmotic homeostasis are paramount to winter survival of hatchling turtles, as in other ectotherms (Ring and Danks, ’94; Block, ’96; Danks, 2000; Williams et al., 2002).

**Water balance**

The water budget of hatchling turtles in terrestrial hibernation is fairly simplistic. They presumably neither drink nor obtain water through feeding during winter, although this supposition requires empirical validation. Unlike amphibians, hatchling turtles reportedly cannot reabsorb moisture from even a saturated substratum through nonoral routes (Costanzo et al., 2001b). Therefore, production of metabolic water may be the sole hydration source (Costanzo et al., 2004).

On the other hand, water readily leaves the body via multiple routes. Some is lost through normal functions of the excretory and digestive systems, although these debits probably are unimportant in the overall water budget. Turtles apparently defecate within a week or two after hatching. Elimination of fecal matter rids the body of potent INA derived from resorbed yolk (Costanzo et al., 2003), as well as bits of eggshell and soil that are consumed during or shortly after hatching (for reasons that remain unclear; see Packard et al., 2001; Costanzo et al., 2003; Packard and Packard, 2006). Micturition is a potentially significant avenue for water loss during hibernation, although little is known about excretory function in hatchling turtles. Changes in blood urea levels during simulated terrestrial hibernation imply that hatching C. picta are anuric or at least refrain from urinating, whereas hatchling C. serpentina, which typically overwinter under water, apparently do void urine (Costanzo et al., 2000b). Generally, urine production ceases in turtles during dehydration (Dantzler and Schmidt-Nielsen, ’66).

Respiration accounts for a small proportion of water efflux when body surfaces are exposed to the atmosphere, but this mode has greater significance when the animal is buried in soil. Management of respiratory water loss in hatchling turtles has received little study. Ventilation rates generally are sensitive to body temperature, although some evidence suggests that these variables can
become uncoupled during cooling. For instance, hatchling *C. picta* show a disproportionately large reduction in ventilatory frequency during cooling at subzero temperatures (Larson, 2004), perhaps reflecting an overall reduction in cellular metabolism as well as increased reliance on anaerobiosis (Hartley et al., 2000; Costanzo et al., 2001a). In addition, the ventilatory pattern is transformed as body temperature falls and, importantly, breathholding can be an effective means to reduce water loss (Schmidt-Nielsen and Bentley, '66; Schmidt-Nielsen et al., '66). According to Larson (2004), hatchling *C. picta* exhibited relatively cyclic and rhythmic ventilation at 15°C, but as the temperature was reduced to ~8°C, turtles began breathing in a discontinuous, noncyclic manner. This shift presumably allowed metabolic adjustments as oxygen demand decreased with cooling; probably it also conserved both water and energy (Seidel, '78; Glass et al., '79).

Evaporation from cutaneous (as opposed to respiratory) surfaces typically accounts for the majority of EWL in reptiles (Bentley and Schmidt-Nielsen, '66; Chessman, '84). Among turtles, carapacial and plastral scutes of the shell better resist water loss as compared with the softer epidermis of the head, neck, and limbs (Rose, '69; Spotila and Berman, '76). Consequently, EWL is influenced by the cutaneous surface area, which, in turn, is governed by shell morphology and body size (Wygoda and Chmura, '90; Stone et al., '92; Stone and Iverson '99). All else being equal, larger turtles have lower mass-adjusted rates of EWL owing to a lower surface-to-volume ratio that favors water conservation. However, no significant association between body size and hibernation habitat has been found among hatchling turtles (Costanzo et al., 2001b).

EWL is also governed by permeability characteristics of the integument (Spotila and Berman, '76); these largely reflect epidermal keratinization (Seidel and Reynolds, '80) and/or lipid content (Roberts and Lillywhite, '80; Lillywhite and Maderson, '88). Cutaneous permeability in turtles has not been extensively studied but probably varies by taxon, life stage, and even acclimatization. Hatchling *C. picta* are highly resistant to EWL (Costanzo et al., 2001b), this trait deriving from a dense epidermal layer of lipid deposits that apparently is lacking in hatchlings of several other species (Willard et al., 2000). During dormancy, *K. flavescens* exhibits reduced rates of EWL, apparently owing to an increase in skin keratinization (Chilian, '76). Among turtles, dehydration tends to suppress EWL (Chessman, '84), as does cold (Olson, '89).

Given the strong influence of morphological attributes and physiology on susceptibility to water loss, it is perhaps not surprising that chelonians vary considerably in this characteristic (Bogert and Cowles, '47; Bentley and Schmidt-Nielsen, '66; Ernst, '68; Chessman, '84; Olson, '89). Mechanisms of water conservation probably are of great significance to species that overwinter terrestrially or in other desiccating habitats. These include behaviors such as retracting the head and limbs into the shell, and in communal hibernacula, forming a cluster with other hatchlings. Measurements of EWL in hatchling turtles are available for few species (Finkler, '99; Costanzo et al., 2001b; Dinkelacker et al., 2004), although the data indicate that terrestrial hibernators generally exhibit lower (mass-standardized) rates of EWL than aquatic hibernators (Table 3). Of particular interest is *E. blandingii*, which exhibits an intermediate susceptibility to water loss.

### TABLE 3. Rates of evaporative water loss (EWL) at 4°C in winter-acclimatized hatchling turtles

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Hibernation habitat</th>
<th>EWL (mg g⁻¹ d⁻¹)</th>
<th>Body mass (g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Terrapene ornata</em></td>
<td>Terrestrial</td>
<td>0.9</td>
<td>10.7</td>
<td>1</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>Terrestrial</td>
<td>1.9</td>
<td>4.0</td>
<td>1</td>
</tr>
<tr>
<td><em>Graptemys geographica</em></td>
<td>Terrestrial</td>
<td>2.0</td>
<td>5.4</td>
<td>2</td>
</tr>
<tr>
<td><em>Kinosternon flavescens</em></td>
<td>Terrestrial</td>
<td>2.4</td>
<td>3.1</td>
<td>1</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Terrestrial</td>
<td>2.6</td>
<td>7.7</td>
<td>1</td>
</tr>
<tr>
<td><em>C. p. dorsalis</em></td>
<td>Terrestrial</td>
<td>3.6</td>
<td>5.0</td>
<td>1</td>
</tr>
<tr>
<td><em>Emydoidea blandingii</em></td>
<td>Unknown, possibly terrestrial</td>
<td>4.1</td>
<td>6.8</td>
<td>3</td>
</tr>
<tr>
<td><em>Chelydra serpentina</em></td>
<td>Aquatic</td>
<td>6.3</td>
<td>8.7</td>
<td>1</td>
</tr>
<tr>
<td><em>Sternotherus odoratus</em></td>
<td>Aquatic</td>
<td>9.6</td>
<td>3.2</td>
<td>1</td>
</tr>
<tr>
<td><em>S. carinatus</em></td>
<td>Aquatic</td>
<td>11.4</td>
<td>2.8</td>
<td>1</td>
</tr>
</tbody>
</table>

Values are means (N = 3–10) reported in the source document. References: (1) Costanzo et al. (2001b); (2) Baker et al. (2003); (3) Dinkelacker et al. (2004).
Although the precise hibernation habits of its hatchlings are unknown, it is possible that they avoid excessive dehydration by hibernating in wet soil in marshes or pond margins (Dinkelacker et al., 2004). Overall, however, the association between EWL and hibernation habitat suggests that dehydration resistance may be an important trait in terrestrial hibernation (Costanzo et al., 2001b).

**Hydric and osmotic stress**

Water influx is the salient osmoregulatory challenge besetting turtles that spend long periods submerged in cold water (Trobe and Stanley, ’71). This phenomenon stems from a continuous osmotic uptake of water and is compounded by an uncompensated salt loss and marked reduction in cardiac output, which allows fluid to pool in underperfused capillary beds (Semple et al., ’70; Stitt and Semple, ’71). Another complicating factor is the inhibiting effect of cold on ion uptake (Dunson, ’67) and urine production (Crawford, ’91a). Although limited renal function in submerged hatchlings probably curbs excretion of salts, it also contributes to water retention.

Few studies of hydro-osmotic balance in submerged hatchling turtles have been published. Turtles actively exchange water and the major ions (except potassium) with their environment, although fluxes in hatchlings apparently differ in some respects from those in adults (Dunson, ’67; Dunson and Seidel, ’86). Limited study shows that the hatchlings of at least some species actively osmoregulate during hibernation in water. During normoxic submergence of hatchling C. picta, shell concentrations of Na\(^+\) and K\(^+\) rose two-fold to three-fold, but this change was not observed in hatchling G. geographica or C. serpentina (Reese et al., 2004b); the significance of these findings remain unclear. Dinkelacker et al. (2005a) studied hydro-osmotic responses in hatchlings of three species of turtles during simulated hibernation in normoxic and severely hypoxic water. E. blandin- gii, C. serpentina, and A. spiniferà varied in their responses, although some generalized patterns emerged. During 75–77 d of normoxic submergence, plasma ion concentrations generally remained static (Na\(^+\), K\(^+\), and Ca\(^{++}\)) or declined (Mg\(^++\)) and, although body water content did not change, plasma osmolality tended to decrease. Responses of hatchlings in hypoxic submergence, which was tolerated for only 6–19 d, were more dramatic. Plasma Ca\(^{++}\) and Mg\(^++\) increased universally, and K\(^+\) also increased in C. serpentina. These changes apparently reflected a mobilization of mineral from the shell, and, together with a marked accumulation of lactate, contributed to an increase in plasma osmolality.

Some evidence suggests that osmoregulation during continuous submergence is stressful, even if the water remains normoxic. Hatchling C. serpentina, which commonly hibernate under water, survived winter (6.5 months) while held at 4°C in water-filled jars (Sims et al., 2001). Over the course of the experiment, plasma osmolality dropped precipitously, from 340 to 290 mosmol kg\(^{-1}\), and the hatchlings lost ~5% of their initial body mass. Lacking data on body water concentration, the authors surmised that increased hydration caused the drop in osmolality; however, an uncompensated salt loss could also have been involved. In contrast, hatchling C. picta succumbed when kept in water-filled jars at 4°C for 70 d (Packard and Packard, 2001c). Unfortunately, there was no attempt to control or measure the ambient PO\(_2\), although the authors suspected ionic-osmoregulatory imbalance was a factor in the turtles’ demise. In a more elaborate experiment, hatchling C. picta (n = 8) were kept over winter in aquaria containing cold, normoxic water, and shelters (J. Costanzo, T. Muir, and B. Dishong, unpublished data). Denied access to air from December–February (simulating ice cover), none died during the 5.5-mo experiment; however, in spring all hatchlings appeared edematous and in poor health, and three were unable to right themselves. Body water concentration in these animals (84.1±0.6% wet mass) exceeded that in control turtles (80.6±0.9%; n = 8) held at the same temperature and for the same duration in simulated terrestrial hibernation; accordingly, their plasma osmolality was also lower (237±4 vs. 351±5 mosmol kg\(^{-1}\), respectively). Differences in dry carcass mass between the groups indicated that the submerged turtles had consumed more energy than the terrestrial hibernators, probably owing to higher costs of managing salts and water.

Little study has been devoted to water relations during the months following hatching; therefore, the extent to which neonates must cope with hydric and osmotic stress in terrestrial hibernation is uncertain. Indeed, some evidence indicates that terrestrial hibernators can remain fully hydrated and in osmotic homeostasis throughout winter. In fact, under favorable environmental conditions, some species actually gain weight that presumably reflects increased hydration (Filoramo...
and Janzen, '99; Costanzo et al., 2000b). Net water gain in terrestrial hibernators could be promoted by a combination of low EWL and a favorable morphology (Costanzo et al., 2001b), the effect of cold on vapor pressure (Olson, '89), and production of metabolic water via β-oxidation of fatty acids. The relatively large lipid and yolk reserves in hatchlings that hibernate terrestrially, as compared with those in aquatic hibernators (Congdon et al., '83c; Congdon and Gibbons, '85, '90; Rowe et al., '95; Nagle et al., '98; Costanzo et al., 2000b), seemingly is adaptive in this regard.

On the other hand, it is clear that turtles can dehydrate while hibernating on land. Christiansen et al. ('85) noted that K. flavescens emerging from hibernacula in sand dunes appeared dehydrated, and hatchling C. picta and T. ornata collected from hibernacula are often—though not always—thirsty (Costanzo et al., 2001b, 2004). The latter observation may be taken as evidence of dehydration because, when placed in water, experimentally dehydrated hatchlings imbibe the quantity needed to restore hydration (Costanzo et al., 2001b). A field study by Costanzo et al. (2004) showed that hydro-osmotic balance in hibernating turtles may be influenced by environmental conditions, especially patterns of precipitation and soil moisture abundance. During an unusually dry winter, hatchling C. picta tended to lose body water and increase plasma levels of urea and other osmolytes, whereas during a wet winter turtles remained in positive water balance and accumulated little urea.

Most reptiles tolerate a considerable degree of desiccation. Turtles, generally, can survive the loss of about one-third of their body water (Hall, '22; Bogert and Cowles, '47; Ernst, '68). Nevertheless, physiological functions are sensitive to the attendant increase in osmotic pressure and ion concentrations, which perturb metabolic and acid–base regulation. In addition, hypovolemia and increased blood viscosity can impair circulatory and gas exchange efficiency. Aside from these direct effects, water loss can impose subtle, deleterious consequences for hatchling fitness and survival, for example, by altering resistance adaptations, such as thermal tolerance (Plummer et al., 2003). Additionally, because locomotor performance of hatchlings is influenced by body hydration (Miller, '93; Miller et al., '93; Finkler, '99; Finkler et al., 2000), excessive water loss can contribute to mortality during overland migration (Kolbe and Janzen, 2001, 2002). Some work suggests that, unless water is discovered en route, hatchlings embarking on a nest-to-water journey in a partially dehydrated state are less successful in reaching their permanent habitat (Tucker and Paukstis, '99).

**Thermal physiology**

Hatchlings of temperate-region turtles are inherently tolerant of cold, irrespective of whether they hibernate on land or under water, and many revive even after chilling to subzero temperatures. Myriad biophysical, physiological, and behavioral responses enable hatchlings to survive temperatures encountered in their hibernacula, although cold tolerance is limited, even in the hardiest species. Behavioral avoidance of extreme cold is the most fundamental and reliable defense against chilling or freezing injury. Avoidance is easily achieved in aquatic hibernators, but hatchlings that overwinter terrestrially, particularly inside nests, have limited ability to evade frost. They therefore depend on either of two mechanisms for surviving transient exposure to subzero temperatures: freeze tolerance or supercooling. Each coping mechanism has its own advantages and disadvantages. For example, although freeze tolerance permits revival after the tissues have frozen and thawed, it offers protection only at modest temperatures. Indeed, cooling of frozen hatchlings below −3 to −4°C apparently is lethal (Storey et al., '88; Churchill and Storey, '92a; Costanzo et al., '95b, 2006; Attaway et al., '98). In contrast, supercooling permits survival over a much broader temperature range (to ~−12°C; Packard and Packard, '99); however, the supercooled state is inherently precarious because turtles are susceptible to inoculation by endogenous and exogenous INA (e.g., Costanzo et al., 2003; Packard and Packard, 2006) and the rapid, uncontrolled freezing of deeply supercooled tissues usually is lethal (Storey and Storey, '88). Freeze tolerance and supercooling usually are dichotomous strategies, but at least one species, C. picta, apparently can benefit from either mechanism, the one invoked during a particular chilling episode depending on physiological and environmental conditions (Lee and Costanzo, '98).

The following sections summarize the literature on cold-hardiness mechanisms of hatchling turtles, highlighting advantages and limitations of each. We caution that our synthesis suffers from ambiguities and uncertainties in the literature. A primary shortcoming is that virtually all such information comes from laboratory experiments.
that may not adequately represent field conditions. In several studies, design flaws stemming from investigators’ failure to observe key aspects of the animal’s physiology or natural history have obfuscated the tenets of cold hardiness, leading to incremental progress in the field. Yet another limitation is that virtually all of the information can be traced to only three research laboratories. Therefore, the literature not only reflects the biases of relatively few investigators, but it also lacks validation through extensive peer scrutiny. Finally, there is virtually no information available for the majority of turtle families, and most of the pertinent literature concerns only one species, *C. picta*. This species is of especial interest because it is broadly distributed over the continent, it (along with *C. serpentina*) occurs at higher latitudes than other North American turtles, and it regularly overwinters within the shallow natal nest, even near the northern limits of its geographic range (Nichols, ’33; Bleakney, ’63; St. Clair and Gregory, ’90; Lindeman, ’91; Koonz, ’98; Rozycki, ’98). Furthermore, it is the subject of a spate of studies attempting to resolve disparate hypotheses about the nature of its cold hardiness (e.g., see commentary by Packard, 2004a). Nevertheless, studies of other turtle taxa, though much less abundant, have greatly helped to elucidate the diversity of environmental conditions and associated survival mechanisms.

**Supercooling**

A cold-hardiness strategy predicated on supercooling permits survival with relatively little physiological stress over a broad range of body temperatures. Reliance on supercooling for freeze avoidance does, however, carry a significant mortality risk. In fact, supercooled solutions are inherently metastable and, although the probability of ice nucleation increases with passage of time and decreasing temperature, freezing of a supercooled solution can occur spontaneously at any temperature below its equilibrium freezing/melting point (Salt, ’66; Sømme, ’82). Spontaneous freezing of a supercooled organism causes ice to form haphazardly and quickly, often with a lethal consequence (Storey and Storey, ’88).

Most organisms can be supercooled to some extent (Andjus, ’55; Lowe et al., ’71; Spellerberg, ’76), although not all organisms can effectively use supercooling as a means to avoid freezing under natural conditions. Indeed, an aqueous medium supercools only if it lacks potent INA that organize water molecules into ice embryos, which then foster rapid, widespread freezing of a solution (Vali, ’95). Furthermore, injury or death from extensive chilling can occur in the absence of freezing. Freeze avoidance through supercooling is an important survival mechanism in a host of ectothermic animals, but is not without potentially deleterious consequences (Lee and Costanzo, ’98).

**Chilling responses and cold tolerance**

How do hatchling turtles behave when exposed to extreme cold? Responses to progressive cooling have been examined in only one study (Costanzo et al., ’99). In both *C. picta* and *C. serpentina*, locomotor activity increased as body temperature approached 0°C, a response also seen in other ectotherms (Costanzo, ’88; Cook, 2004), but declined as temperature was further reduced. Ultimately, all turtles became immobile, but there were some important differences between the species. The terrestrial hibernator, *C. picta*, retained some neuromotor functions at temperatures as low as −10°C; *C. serpentina*, which occurs in the north but usually hibernates in thermally buffered aquatic sites, entered cold rigor at −5°C. This work also revealed that *C. serpentina* from a southern population became immobilized at temperatures higher than those impacting northern conspecifics, suggesting that natural selection has favored increased cold tolerance in the latter (Costanzo et al., ’99).

Hatchling turtles are remarkably tolerant of being chilled in the unfrozen state to very low temperatures. Critical thermal minima have not been determined for many species, although *C. picta* reportedly can recover from acute chilling to temperatures as low as −10 to near −15°C (Packard and Packard, ’93b, ’99; Costanzo et al., ’99, 2000a) and *C. serpentina* to at least −4 or −5°C (Packard et al., ’93; Costanzo et al., ’99). Hatchling *C. picta* reportedly recover from supercooling for 11 d at −10°C (Packard et al., ’99a), for 25 d at −8°C (Hartley et al., 2000), and for 30 d at −5°C (Packard and Packard, ’97). In both *C. picta* and *C. serpentina*, cardiac contractions continue at body temperatures approaching −8°C, although the heart rate is greatly reduced (Birchard and Packard, ’97). Even *C. picta* from the southern limit of its geographical distribution can tolerate 8 d of continuous supercooling, with a critical minimum temperature near −9°C (Packard et al., 2002).
It was long presumed that supercooling was benign (Lowe et al., '71; Spellerberg, '72; Halpern, '79; Costanzo and Lee, '95), but recent studies have shown that extended hypothermia and acute exposure to severe cold can impair physiological functions. In hatchling turtles, diminished cardiac output and tissue perfusion at subzero temperatures (Birchard and Packard, '97) results in lactate accumulation and, presumably, reduced intracellular pH (Hartley et al., 2000; Costanzo et al., 2001a; Baker et al., 2003). Consequently, species that poorly tolerate hypoxia may be unable to use supercooling as a winter survival strategy. It is conceivable that supercooled tissues generate ROS and suffer oxidative stress. However, recent work (Baker et al., 2007) suggests that tissue levels of protein carbonyls and thiobarbituric acid reactive substances (TBARS), products of oxidative damage to proteins and lipids, respectively, do not increase during 24-h recovery for hatchling C. picta held supercooled at −6°C for 48 h. Oxidative stress resulting from supercooling and subsequent rewarming has not been examined in hatchlings of other species, but apparently is absent in the cold-hardy common lizard (Lacerta vivipara), perhaps because they increase activities of certain antioxidant enzymes (Voituron et al., 2005b). The hatchlings of some turtle species maintain high constitutive levels of antioxidants (Dinkelacker et al., 2005b; Baker et al., 2007) and thus are in a constant state of preparedness.

Extended chilling and exposure to severe cold can result in mortality, even if tissues never freeze. In one study (Burke et al., 2002), Italian wall lizards (Podarcis sicula) reanimated quickly following experimental chilling to between −2.4 and −3.7°C, but an individual supercooled to −16°C did not revive. Extreme chilling can also be injurious or lethal to hatchling turtles. Mortality in hatchling C. picta occurs when turtles are cooled to between −11 and −13°C (Packard and Packard, ’93b), and none revive after being supercooled even briefly to −15°C (Packard and Packard, ’99). Some evidence suggests that chill tolerance depends on season or stage of development. For example, winter-conditioned C. picta hatchlings survived exposure to −10°C for at least 24 h (Packard and Packard, ’93b), whereas animals tested in spring had poor survival when held at −10°C for as little as 5 h (Packard and Packard, ’99). Furthermore, recently hatched, unacclimatized C. picta died when chilled at −2°C for 7 d (Packard and Packard, 2003a). Apparently, temperature and exposure duration interactively determine chill tolerance. In one study, hatchling C. picta recovered after being held at −8°C for 5 or 15 d, but high mortality resulted after 25 d (Hartley et al., 2000).

The literature maintains that hatchling turtles can survive being supercooled for prolonged periods so long as they avoid extreme cold. Thus, it is noteworthy that mortality has occurred in hatchling C. picta exposed only to −4 or −7°C (Packard and Packard, ’97), and even −2°C (Packard and Packard, ’95a). Unfortunately, the experimental methodology in these studies does not preclude the possibility that the succumbing turtles actually froze, unknown to the authors. In fact, Packard and Packard (2003b) suspected that they failed to detect the freezing of C. picta that died after being exposed to −2°C for 7 d.

The aforementioned studies focused on survival in response to subzero temperature exposure. Some information suggests that physiological impairment can occur at temperatures above those that prove lethal. Hatchling C. picta promptly recovered from being held supercooled at −4°C for 5 d; on rewarming, they responded normally to tactile stimulation and burrowed into the substratum (Costanzo et al., 2001a). In contrast, hatchlings held supercooled at −6°C for the same period required 3 d to fully recover and were initially lethargic, unresponsive to stimulation, and remained on the soil surface. Packard and Packard (’93b) reported that hatchling C. picta supercooled to −12°C, a near-lethal temperature, were slow to recover normal behavioral functions, but ultimately became active and fed.

What are the proximal cause(s) of chilling injury? Damage might stem from adverse lipid phase transitions and their deleterious effects on membrane structure and function (Drobnis et al., ’93; Kostal et al., 2003; Tomcala et al., 2006). Consistent with this hypothesis, recent investigations with hatchling turtles have shown that injury is induced over a narrow thermal range (J. Costanzo and T. Muir, unpublished data). In these experiments, hatchling C. picta (n = 20) tolerated exposure to −10.5°C for 10 h, but no turtles (n = 20) recovered from a 24-h exposure to −13°C. Turtles in the latter group had beating hearts and patent circulation, but did not respond to tactile stimulation; thus, the nervous system may be a primary site of chilling injury (see Costanzo et al., ’99). Because nervous tissues respond to thermal adaptation over both brief

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and evolutionary time periods (Macdonald, ’81), one would expect to find variation in chilling tolerance among hatchlings of species with diverse life histories. Notably, although C. picta tolerated exposure to −10.5°C, neither M. terrapin (n = 4) nor T. scripta (n = 5) recovered, suggesting that the former is particularly well adapted to survive extreme cold. The basis of its enhanced cold hardiness remains to be elucidated. In hatchling C. picta, cell membranes reportedly (DePari, ’88) have a higher percentage of unsaturated fatty acids than in hatchling C. serpentina, a feature that presumably maintains membrane fluidity and prevents deleterious phase transitions at lower temperatures (Guschina and Harwood, 2006).

One of the most instructive findings of Costanzo and Muir (unpublished data) was that chilling tolerance was sensitive to thermal history. In one experiment, hatchling C. picta were preconditioned by cooling them at −2°C d⁻¹ until they had reached −7°C, holding them at this temperature for 2 d, and then passively warming them to 0°C. Transient exposure to this intermediate temperature markedly improved their chill tolerance, as 10 of 20 hatchlings survived an ensuing 24-h exposure to −13°C, an otherwise lethal treatment. This result is reminiscent of the rapid cold-hardening phenomenon exhibited by various arthropods, and could derive from remodeling of cell membranes (Kostal et al., 2003; Overgaard et al., 2005, 2006; Lee et al., 2006a) and myriad changes in the metabolite profile (Bohn et al., 2007). Stress-induced thermoprotection can also stem from upregulation of stress proteins, such as Hsp 70, which protect against cytoskeletal perturbation, among other functions (Klose and Robertson, 2004; Robertson, 2004). Cold preconditioning may also improve the organism’s ability to maintain transmembrane ion gradients (Kostal et al., 2004), perhaps by suppressing ion channels. Whether or not chilling injury is an important natural cause of winter mortality remains to be determined. Also unknown is whether individuals surviving periods of supercooling can later exhibit diminished performance and fitness, as has been demonstrated for other organisms (Layne and Kuharsky, 2001).

**Methodological considerations**

The usual laboratory method for determining supercooling capacity is to monitor the body temperature of individuals during progressive chilling in a refrigerated bath, noting the temperature at which body fluids spontaneously freeze. The ensuing phase transition is marked by an abrupt release of the latent heat of fusion, which is detected by a thermocouple placed on, or immediately adjacent to, the specimen. Note that, although internal placement of the probe gives a more accurate measure of core body temperature, this approach should be avoided because it can initiate freezing. The lowest temperature recorded before the exotherm appears, the temperature of crystallization (Tc), represents the limit of supercooling under the particular experimental conditions. The Tc is equivalent to the supercooling point; however, the latter term, an unfortunate literature convention, erroneously implies that the measured value is an invariable, phenotypic attribute (sensu Lowe et al., ’71).

If the experimenter’s aim is to determine innate supercooling capacity, then experimental subjects must be isolated from dust, soil, and other potent INA, both before and during testing. Subjects must also be free of surface moisture, which can freeze and inoculate tissues. For hatchling turtles, this requirement can be met by keeping them on clean vermiculite, a common incubation medium that is sterilized during its manufacture and lacks significant ice nucleation activity (Costanzo et al., 2000a). Before testing, hatchlings are cleaned and permitted to air dry before being chilled in a clean, dry environment. Cooling rates used in supercooling trials have not been standardized (Costanzo et al., 2000a; Packard et al., 2001).

The Tc values determined for specimens maintained and tested in isolation from ice and INA represents the maximal limit of supercooling under idealized conditions. Packard and coauthors (’95a) stated that refraining from thoroughly cleaning and drying hatchling C. picta rendered them “extraordinarily prone to freezing at high subzero temperatures” in supercooling trials. Even brief contact with nesting soil harboring native INA can markedly constrain supercooling capacity. Not surprisingly, the limit of supercooling for C. picta hatched and reared on natural substrata typically is 5–6°C higher than that determined for vermiculite-reared hatchlings (Costanzo et al., 2000a; Packard et al., 2001). Consequently, inter-study comparisons are confounded by inconsistencies in experimental protocol, which can lead to erroneous conclusions. In particular, the observation that supercooling capacity is greater in hatchlings near the northern
limit of their range (Packard and Packard, '95a; Packard et al., '97b; Costanzo et al., 2000a) than in hatchlings from several more temperate locales in the eastern United States (DePari, '88; Claussen and Zani, '91; Packard and Janzen, '96) and southern Canada (Storey et al., '88, '91; Churchill and Storey, '92a) has promulgated the idea that this species exhibits adaptive geographic differences in supercooling capacity. However, despite its attractiveness from an evolutionary perspective, the variation simply reflects different methods of rearing hatchlings, which may or may not have become contaminated with INA (for a more extensive discussion, see Costanzo et al., 2000a). Results of laboratory supercooling trials must be carefully interpreted in the context of the experimental protocols used by the investigators.

Morphological and physiological influences

With the approach of winter, many temperate ectotherms undergo physiological changes that improve cold hardiness. Biochemical and physiological mechanisms promoting development of cold hardiness are best known among invertebrates (Lee, '91; Loomis, '91; Oswood et al., '91; Duman et al., '95). With the exception of certain coldwater fishes (DeVries, '82), evidence for seasonal variation in cold hardiness among ectothermic vertebrates is scanty (Costanzo and Lee, '95). Nevertheless, several laboratory studies (Costanzo et al., 2000b, 2003; Packard et al., 2001) of hatchling turtles have documented a profound increase in supercooling capacity coincident with winter acclimatization. In these investigations, C. picta reared on (INA-free) vermiculite and tested soon after hatching supercooled modestly ($T_c$, −6 to −11°C); however, following cold acclimation they supercooled to near −17°C. This change, induced by elimination or masking of endogenously produced INA (see below), is striking in vermiculite-reared animals and gives the illusion that these hatchlings could avoid freezing in even the coldest winter. To the contrary, C. picta reared on native nesting soil (rather than vermiculite) exhibit a relatively modest gain in supercooling capacity (e.g., 2–5°C) with cold acclimation (Packard et al., 2001; Costanzo et al., 2003; Packard and Packard, 2003a, 2006). These turtles, which are irreversibly contaminated with natural INA, exhibit mean $T_c$ values that range from −10 to −7°C, even when fully cold-hardened and cooled in isolation from ice and environmental INA (Costanzo et al., 2000a, 2001c, 2003; Packard et al., 2001; Packard and Packard, 2003a, 2006). Moreover, hatchlings collected directly from natural hibernacula supercool on average to only −7 or −8°C (Costanzo et al., 2001c, 2003; Packard et al., 2001).

Organisms obey physical laws governing solutions; therefore, it is not surprising that many ectotherms, including reptiles, are capable of supercooling deeply (Lowe et al., '71; Spellerberg, '72). Because the various INA in a solution are randomly distributed, and the probability that a given solution contains an efficient, high-temperature nucleator increases with volume (Vali, '95), small organisms generally can be supercooled more extensively than large ones (Costanzo and Lee, '95; Lee and Costanzo, '98; Wharton, 2002; Ansart and Vernon, 2003). Accordingly, hatchling turtles (and perhaps some small lizards—Halpern, '79; Costanzo et al., '95a) are among the few vertebrates that can reliably use supercooling as a winter survival mechanism. Claussen and Zani ('91), in an investigation of allometric variation in adult and hatchling C. picta, found no correlation between supercooling capacity and body size; however, the relatively high $T_c$ values reported for all individuals suggest that nucleation was triggered by a thermocouple probe placed in the intestine. In a comparative study of hatchling turtles, Costanzo et al. (2000a) found that, after excluding the exceptionally low $T_c$ values obtained for C. picta, smaller turtles supercooled more extensively than larger individuals, irrespective of taxon. However, in a subsequent study (Costanzo et al., 2001b), variation in body size (range in mean body mass, 3–11g) did not appreciably influence supercooling capacity in hatchlings of eight taxa. All of the specimens used in that study were relatively small, however. The authors concluded that body size, per se, probably does not preclude the use of supercooling as a winter survival mechanism in hatchling turtles.

The amount and distribution of water within an organism may strongly influence supercooling capacity, and some ectotherms seasonally undergo a partial dehydration that enhances their cold hardiness (Zachariassen, '91; Block, '96; Lee and Costanzo, '98; Danks, 2000). This effect at least partly reflects the lowered equilibrium freezing/melting point (owing to solute concentration) and reduced body fluid volume. Experimental dehydration apparently enhances supercooling capacity in hatchling C. picta (Costanzo et al., '95b). These turtles can dehydrate considerably during
winter conditions increased plasma osmolality.

In a comparative study, Costanzo et al. (2001b) determined that marked variation in the body water content (range, 76–83% of fresh body mass in eight taxa) did not explain interspecific variation in supercooling capacity, but neither did having high body water content preclude the use of supercooling as a winter survival mechanism.

Antifreeze proteins (AFPs), also known as “thermal hysteresis factors” and “ice-structuring proteins,” occur in various organisms relying on supercooling to survive exposure to subfreezing temperatures (DeVries, ’82; Duman et al., ’91, ’95). In blood samples, these highly specialized peptides and glycopeptides arrest the growth of ice embryos at temperatures as low as 2–6°C below the equilibrium freezing/melting point. However, functioning in vivo, they may prevent freezing over a much larger range of temperatures (Zachariassen and Husby, ’82). Antifreeze compounds play a critical role in the winter survival of some polar fishes (DeVries, ’82), but their importance in cold hardiness of hatchling turtles (and other vertebrates) is equivocal. They apparently are absent from the blood of freeze-tolerant wood frogs, Rana sylvatica (Wolanczyk et al., ’90), hatchling C. picta (Storey et al., ’91; Costanzo et al., 2000b), and hatchling C. serpentina (Costanzo et al., 2000b). It is unknown whether they occur in turtles, such as hatchling G. geographica, which survive extreme chilling exclusively by freeze avoidance (Baker et al., 2003). Furthermore, it is possible that AFPs are absent from the blood but nevertheless occur in extravascular compartments or intracellular spaces (Olsen et al., ’98).

As a rule, increasing the concentration of osmotically active solutes in a solution colligatively enhances its capacity to supercool. Many freeze-avoiding invertebrates exploit this principle by accumulating compatible osmolytes, such as polyols, sugars, and certain amino acids, and/or by shedding a portion of their body water (Storey and Storey, ’88; Lee, ’91; Ansart and Vernon, 2003). The question of whether freeze-avoiding turtles (and other ectothermic vertebrates) also use this mechanism has received little study. Costanzo et al. (2000b) showed that acclimation of hatchling C. picta to simulated winter conditions increased plasma osmolality markedly (by 60–70 mosmol L−1), much of the increase being attributed to a two-fold to three-fold increase in urea, which in fully cold-acclimated animals comprised 20% of the osmotically active solutes. In contrast, hatchling C. serpentina, which do not supercool as extensively as hatchling C. picta, showed a decrease in plasma osmolality with a concomitant reduction in tissue urea. In another study, urea was found to be a major organic osmolyte in winter conditioned hatchlings of various turtle taxa (Costanzo et al., 2006).

In some organisms, certain amino acids may function as compatible osmolytes (Somero and Yancey, ’97). The amino acid pool in hatchling C. picta is maintained during cold acclimation, and slight increases are seen only in serine and tyrosine (Costanzo et al., 2000b). Similarly, cold acclimation has little effect on the quantity of glucose or carbohydrates that in many insects and other ectotherms contribute to increased osmotic pressure. Glycerol, mannose, sorbitol, erythritol, threitol, and fructose are absent or are present in low concentrations in cold-acclimated hatchling C. picta (Storey et al., ’88; Churchill and Storey, ’92a; Costanzo et al., 2000b). High-performance liquid chromatography analysis of plasma indicated that cold acclimation is associated with a 50–70% increase in plasma concentration of inositol and de novo production of six unidentified carbohydrates (Costanzo et al., 2000b); however, given their very low concentrations, it seems unlikely that they could enhance supercooling capacity appreciably. In fact, it is doubtful that the colligative effect of this change can account for the exceptional supercooling capacity of winter C. picta (Zachariassen and Hammel, ’76; Lee et al., ’81).

**Taxonomic and ecological associations**

Although the available literature suggests that supercooling capacity in hatchling turtles varies taxonomically (Table 4), comparing data among studies is risky because methods of incubation, housing, acclimation, and testing strongly influence the outcome of supercooling trials. Two investigations examined taxonomic variation in this character, both ensuring that all specimens were reared on (INA-free) vermiculite, acclimated to 4°C, and cooled in the absence of external ice and INA (Costanzo et al., 2000a, 2001b). In the earlier report, a terrestrial hibernator, C. picta, froze at −17.8°C, ~7–12°C below the Tc recorded for three aquatic hibernators, S. odoratus, A. spinifera, and C. serpentina. In the latter

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project, mean \( T_c \) varied among eight taxa, ranging from \(-8.1\) to \(-15.5^\circ C\). These studies showed that the supercooling limit for each species more or less reflected the thermal extremes it encounters in its particular winter microenvironment. For example, the terrestrial hibernators, \( T. \ ornata, \ T. \ scripta, \) and \( C. \ p. \ bellii, \) supercooled extensively, whereas aquatic hibernators, \( C. \ serpentina, \ S. \ odoratus, \) and \( S. \ carinatus, \) supercooled only modestly. Supercooling capacity was also less developed in \( K. \ flavescens, \) a species that hibernates deep in the soil, below the reach of frost (Costanzo et al., ’95b).

Other studies, conducted in the same laboratory using identical methods, documented extensive supercooling (mean \( T_c \), \(-14\) to \(-15^\circ C\)) in hatchling \( G. \ geographica \) (Baker et al., 2003), \( M. \ terrapin \) (Baker et al., 2006), and \( E. \ blandingii \) (Dinkelacker et al., 2004), species known or suspected to hibernate terrestrially. Supercooling capacity in \( C. \ picta \) exceeds that of other turtle species and most other similarly sized ectotherms (Lee and Costanzo, ’98). One hatchling froze only after reaching \(-20^\circ C\); this value is the lowest \( T_c \) reported for any vertebrate and is on par with that of a water droplet (Costanzo et al., 2000a). The basis for the exceptional intrinsic supercooling capacity of hatchling \( C. \ picta \) remains unknown.

Although turtle species clearly vary in their intrinsic capacity to supercool, the underlying causes of such variation are incompletely understood. Body size and body water content influence supercooling limits in some organisms, but these factors probably explain little of the observed variation among hatchling turtles, as neither varies appreciably among species (Costanzo et al., 2001b). Certain species apparently are more proficient than others at masking or eliminating internal INA. For example, although the blood plasma of hatchling \( C. \ serpentina \) can be supercooled to \(-12^\circ C\) (Costanzo et al., 2000b), intact animals invariably freeze at much higher temperatures (Costanzo et al., 2000a, 2001b), perhaps because this species retains INA in extravascular spaces, even after cold acclimation. By contrast, the limit of supercooling for plasma of winter-acclimatized \( C. \ picta \) more or less matches that of the intact organism. Perhaps some species enhance supercooling capacity through behavioral mechanisms, such as remaining quiescent during cooling (Costanzo et al., ’99), although this idea remains conjectural.
Regulation of ice nucleation

Principles of biological ice nucleation, including those germane to overwintering in hatchling turtles, have been summarized (Costanzo and Lee, '95, '96; Lee and Costanzo, '98). Organisms depending on supercooling for winter survival must manage the risk of freezing by minimizing the time they spend at subzero temperatures and by limiting their exposure to excessive cold. More critically, they must remain isolated from all entities that can trigger ice nucleation in body fluids at high subzero temperatures. These hazards include ice itself and various INA of endogenous and environmental origins. To safely exploit the physical phenomenon of supercooling, organisms must be capable of masking or eliminating internal INA and mounting barriers to ingress of external ice and INA.

Regulation of ice nucleation is also of great importance to freeze-tolerant organisms, but for a very different reason. Survival of any given freezing episode requires that inoculation be controlled in a manner that prevents intracellular ice formation and permits ice to propagate slowly (Storey and Storey, '92; Duman et al., '95; Zachariassen and Kristiansen, 2000). Accordingly, many species employ one or more mechanisms that initiate freezing at a temperature close to the equilibrium freezing/melting point of body tissues. Three potential strategies for achieving nucleation control, in increasing order of efficacy and reliability, include endogenous synthesis of INA, active incorporation of environmental INA, and inoculative freezing through intimacy with environmental ice and INA. The diversity of nucleation-regulation mechanisms employed by freeze-avoiding and freeze-tolerant hatchlings is an interesting and multi-dimensional feature of their winter biology.

Endogenous INA

Seasonal cold hardening in freeze-avoiding ectotherms commonly involves elimination of ingested food and other matter that could trigger ice nucleation (Sømme, '82; Zachariassen, '85; Bale et al., '89; Duman et al., '95). Similarly, for hatchling turtles, achieving the full capacity to supercool requires purging the gut of potent INA. *C. picta* hatched and reared on vermiculite, an essentially INA-free substratum, initially freeze at relatively high temperatures (−6 to −11°C), but develop a more extensive supercooling capacity during winter acclimatization, coincident with removing or masking endogenous INA (Costanzo et al., 2000b, 2003; Packard et al., 2001). Costanzo et al. (2000b) determined that the blood plasma of neonates lacks ice-nucleating activity and deduced that the INA are restricted to intracellular and/or interstitial compartments. Further study (Costanzo et al., 2003) showed that the neonatal gut harbors INA that, derived from internalized yolk, are eliminated in feces during the weeks following hatching. Factors initiating gut evacuation apparently include maturational changes, given that some individuals defecated within a few weeks of hatching, even before ambient temperature was reduced. Collectively, these studies suggest that seasonal development of cold hardiness depends on elimination of endogenous INA and an unmasking of innate supercooling capacity (Fig. 6).

One perplexing discovery in this line of investigation was that *C. picta* commonly ingested substratum and bits of eggshell during, or shortly after, hatching (Packard et al., 2001; Costanzo et al., 2003; Packard and Packard, 2006). This matter is not retained throughout hibernation, but is voided with feces during the weeks following hatching. The significance of this behavior is unknown, although presumably it could aid gut elimination (e.g., roughage), inoculate the tract with normal flora, or otherwise promote proper gut function.

On the other hand, ingesting external matter seemingly predisposes hatchlings to freezing risk. Nesting soil contains at least two classes of INA,
including various mineral particulates (insoluble, largely unaffected by high temperature and pressure) and organic entities (water-soluble, autoclave-sensitive) that apparently derive from certain microorganisms (Costanzo et al., 2000a). Feeding a small quantity of either type to hatchling

\( C. \textit{picta} \) severely limits supercooling, as mean \( T_c \) rises from \(-17^\circ C\) to \(-7^\circ C\) (Fig. 7). Dissecting the intestine attests that turtles hatched on nesting soil ingest, but ultimately eliminate, particulate INA (Costanzo et al., 2003; Packard and Packard, 2006). However, turtles long retain organic INA, which, in the case of microbes, may have colonized the gut. Consequently, any gain in supercooling capacity associated with purging the gut is slim, as, for example, the limit of supercooling measured in winter \( C. \textit{picta} \) was only \(~2^\circ C\) lower than that measured in autumn, \(-6^\circ C\) (Packard et al., 2001).

Collectively, these studies indicate that contamination by soil INA, either by ingestion or some other route, is a serious constraint on supercooling capacity and, further, raise pointed questions about the efficacy of supercooling as a cold-hardiness strategy in terrestrially hibernating turtles. Experiments with \( C. \textit{picta} \) reared on (INA-free) vermiculite and meticulously groomed and dried before testing have led authors to conclude that “supercooled turtles exposed to temperatures spanning the range recorded in natural nests are at virtually no risk of freezing when they are removed from contact with crystals of ice” (Packard and Packard, ’95a). This contention may be true of specimens kept in a sterile environment, but seems untenable for turtles in nature. Indeed, studies of field-collected animals confirm that fully cold-hardened hatchlings can supercool to only \(-7^\circ C\) or \(-8^\circ C\) (Costanzo et al., 2001c, 2003; Packard et al., 2001).

For most freeze-tolerant organisms, it is critical to survival that ice nucleation occurs at a temperature near the tissue equilibrium freezing/melting point. Many such species use extra-cellular INA, often synthesized during seasonal cold hardening, to control ice nucleation (Duman et al., ’95; Lee and Costanzo, ’98; Zachariassen and Kristiansen, 2000). These agents include both inorganic crystals and specialized proteins, the latter being more common. Ice-nucleation proteins, which have been characterized for several invertebrates, apparently organize water molecules through hydrogen bonding to form an ice embryo.

Agents exhibiting similar characteristics have been found in the blood of freeze-tolerant vertebrates, including \( C. \textit{picta} \) (Wolanczyk et al., ’90; Storey et al., ’91), but their adaptive significance has been challenged for several reasons (Costanzo and Lee, ’96). One relevant issue is that they nucleate blood plasma in vitro at \(-7\) to \(-8^\circ C\), several degrees below the lower limit for freezing survival. In addition, under idealized laboratory conditions, hatching \( C. \textit{picta} \) can supercool well below \(-8^\circ C\), so the activity of these agents is not expressed in vivo. Finally, similar ice-nucleating activity is also found in the blood plasma of various freeze-intolerant species (Costanzo and Lee, ’95, ’96). Costanzo et al. (2000b) determined that the activity was an artifact of sample contamination with exogenous INA and confirmed that the plasma of hatching \( C. \textit{picta} \) (and \( C. \textit{serpentina} \)) lacks ice-nucleation proteins. Additional investigation is needed to determine whether specialized nucleating proteins are found in the blood (or perhaps on cell membranes; see Izumi et al., 2006) of any turtle taxa. Such studies might profitably focus on freeze-tolerant species, such as \( M. \textit{terratin} \), whose ability to supercool is relatively poor.

### Inoculative freezing

Eliminating endogenously produced and ingested INA from the body provides no guarantee
that an organism will remain supercooled; freezing can still occur through seeding by environmental ice or INA (Salt, '63; Ansart and Vernon, 2003; Holmstrup, 2003). Among terrestrially-hibernating turtles, inoculative freezing often is triggered by contact with frozen soil or other substrata at relatively high temperatures (Packard and Packard, '93a, b, '95a, '97, 2003b; Packard et al., '93, '97b; Costanzo et al., '95b, '98, 2000b, 2001b, c; Willard et al., 2000; Baker et al., 2003, 2006; Dinkelacker et al., 2004). Ice is a particularly potent catalyst, but inoculation can result from contact with various inorganic and organic INA ubiquitously found in terrestrial hibernacula (Costanzo et al., 2000a). Susceptibility to inoculative freezing is governed by temperature and a host of environmental factors that mediate an organism's intimacy with these agents (Costanzo and Lee, '96; Lee and Costanzo, '98). It is also influenced by behavior, morphology, and physiology, and thus varies markedly among taxa and, potentially, within populations of conspecifics (Costanzo et al., 2001b).

Physical events precipitating inoculative freezing in ecotothermic animals are incompletely understood. In some invertebrates, ice apparently infiltrates the body through respiratory pores, exoskeletal joints, gill lamellae, and body orifices (Salt, '63; Lee et al., '96; Frisbie and Lee, '97; Holmstrup, 2003; Issartel et al., 2006). Amphibians are highly susceptible to inoculative freezing owing to the ease at which ice propagates across moist, permeable skin (Layne et al., '90; Layne, '91). The less permeable integument of reptiles and fishes better resists ice transfer (Valerio et al., '92; Costanzo and Lee, '96), although turtle skin is not a perfect barrier, as experimentally wetted hatchlings are readily inoculated when the moisture on their surface freezes (Attaway et al., '98; Packard et al., '99b; Packard and Packard, 2004; Costanzo et al., 2006). This fact has biological relevance when, owing to differential patterns of heating and cooling between turtles and the surrounding soil, water vapor condenses on the surfaces of terrestrially hibernating turtles (Thompson, '88).

Some work indicates that inoculative freezing can occur even if a hatchling avoids physical contact with ice. Interpreting the seminal work by Salt ('63), Packard and Packard ('93a) proposed that minute ice crystals grow in a plume of water vapor emanating from integumentary "pores," ultimately transmitting across the skin and triggering global freezing of supercooled body fluids. This scenario could explain why, in some species, inoculation is not necessarily instantaneous, but may be delayed hours or days after initial exposure to environmental ice and INA (Packard and Packard, '93a, '95a, '97; Costanzo et al., '95a; Packard et al., '97b, '99a, 2002).

Much of the literature concerning inoculative freezing in hatchling turtles has addressed the mediating role of the skin, perhaps suggesting that the integument is the sole avenue for INA transmission (e.g., Packard and Packard, '93a,b, '95a, 2003b; Packard et al., 2000; Willard et al., 2000; Packard, 2004a). However, it is important to realize that ice and/or INA can also access body fluids through ingestion and by way of the ocular and otic openings, nares, cloaca, and umbilicus (Kalabukhov, '58; Lowe et al., '71; Spellerberg, '72; Costanzo et al., 2000a, 2001b, 2003). Empirical evidence for inoculation through these avenues is scanty, although manual application of ice to the umbilicus or skin of a supercooled turtle promptly triggers freezing (Packard and Packard, '93a; Packard et al., '93, '97c). That freezing can be initiated by contact with inorganic INA (e.g., sand grains and other particulates) too large to penetrate skin pores (Costanzo et al., 2000a) implies that INA can invade through nonintegumentary routes. Several different routes may admit INA, as activity is found in some, but not all, body compartments (Costanzo et al., 2003). Whether INA ultimately access and nucleate the tissues depends, in part, on the relative sizes of the agents and the external openings (Gehrken, '92; Valerio et al., '92).

Some authors ardently maintain that hatchling turtles can fully "resist" inoculative freezing, being essentially immune to this peril (Packard and Packard, 2001a; Packard, 2004a). A competing view is that any hatchling achieving sufficient intimacy with environmental ice or INA is destined to freeze, and, furthermore, that ice nucleation via this process can occur at any temperature equal to or below the tissue equilibrium freezing/melting point (Costanzo et al., '95b). Indeed, the fact that hatchlings are highly susceptible to direct physical contact with ice (but see Packard and Packard, '93a) underlies the common method used to control ice nucleation in freeze-tolerance trials (Packard et al., '99b; Packard and Packard, 2004; Costanzo et al., 2006). However, as we discuss below, inoculative freezing is not necessarily inevitable, and whether or not it occurs during a given chilling episode depends on exposure duration, various physiological and
environmental factors, and, to some extent, stochasticity.

**Methodological considerations**

Generally, inoculative freezing is studied in the laboratory by progressively cooling specimens in the presence of ice and/or INA until their body fluids freeze. The resultant $T_c$, measured with a thermocouple placed near each specimen, is taken as a measure of the capacity for freeze avoidance achieved under the specific conditions of the trial. Although the procedure is simple, designing meaningful experiments presents several technical challenges. First, it is important to configure the experiment so that the study subjects are exposed in a naturalistic manner to the kinds and amounts of INA prevalent in the hibernaculum microenvironment. It is also important to ensure that the exposure occurs over ecologically relevant time periods and temperature ranges.

Studies of inoculation resistance in hatchling turtles commonly involve cooling hatchlings in a simplified representation of a terrestrial hibernaculum. In practice, a turtle is placed in a vessel and fully immersed in an appropriate substratum. A thermocouple, used for tracking temperature during cooling and registering the freezing exotherm, is positioned adjacent to the hatchling. If the objective concerns inoculative freezing by environmental ice, the substratum should be frozen before the subject is allowed to become supercooled. This is achieved simply by inoculating the soil with a few ice crystals and allowing it to freeze at a temperature slightly above the equilibrium freezing/melting point of turtle tissues. Once the soil solution has thoroughly frozen, the entire system is progressively cooled until the thermocouple registers a freezing exotherm, yielding the $T_c$ of the turtle within.

The methodology described above, by virtue of its simplicity, can be standardized so that data for different experimental groups, taxa, etc. are comparable. It can determine fairly precisely the lowest temperature each specimen attained before freezing, and this metric is a useful gauge of freezing resistance capacity under the conditions of the trial. On the other hand, it is a poor facsimile of the actual hibernaculum microenvironment, which is far more complex, both physically and thermally. Natal nest hibernacula, for example, commonly include other hatchlings, broken eggshells, various organic debris, and possibly an air space. Given these limitations, results of such trials should be interpreted cautiously. It is important to realize that experimental outcomes are highly specific to the testing conditions used, and, unless a standardized protocol is followed, results of different studies should not be directly compared.

Another important consideration is that erroneous conclusions may be drawn if conditions of inoculation trials are inappropriate to the biology of the subjects under investigation. For example, measures of inoculation resistance for *C. picta* hatched and reared on a (INA-free) vermiculite, meticulously groomed to remove moisture and debris, and tested in a non-indigenous clayey soil (e.g., Packard and Packard, '93a,b, '95a; Packard and Janzen, '96; Packard et al., '97c) undoubtedly overestimate the ability of these hatchlings to supercool in nature. Overestimates also result from the practice of “head-starting”—i.e., cooling hatchlings in a substratum that for a time remains supercooled and, therefore, lacks ice crystals (Packard and Packard, '90, '93a,b, '95a, '97; Packard et al., '93, '97c, 2000; Costanzo et al., '95b; Packard and Janzen, '96; Willard et al., 2000). Failure to freeze the substratum before initiating the trial also creates a serious technical problem in that the large, protracted exotherm produced when the substratum eventually freezes can wholly eclipse the relatively minor signal from the hatchling, thus making it impossible to determine whether the animal has actually frozen. Although this difficulty is easily avoided by using the smallest volume of soil that fully envelops the hatchling (e.g., Costanzo et al., '98), exotherm “masking” is a recurring, confounding issue in many reports (Packard and Packard, '90, '93a,b, '97, 2003b; Packard et al., '93, '97c, 2000; Willard et al., 2000).

**Morphological and physiological influences**

Freeze-avoiding organisms commonly exhibit a seasonal development or enhancement of inoculation resistance that is timed to provide maximum protection against (lethal) freezing during the coldest weather (Fields and McNeil, '86; Lee, '91; Gehrken, '92; Rojas et al., '92). This response is also exhibited by hatchlings of some turtle species (Fig. 8). In laboratory studies, recently hatched *C. picta* were highly susceptible to inoculation, whereas cold-acclimated hatchlings supercooled more extensively in the presence of environmental ice and INA (Costanzo et al., 2000b; Packard and...
Packard, 2003b). Data for C. picta collected from natural hibernacula at intervals from fall through early spring corroborate these laboratory findings (Costanzo et al., 2004). Such variation implies that inoculation resistance is under morphological and/or physiological control, although the underlying mechanisms have not been elucidated.

Ultrastructural characteristics of the integument have important influences on the skin’s role as a barrier between the internal and external milieus. In dormant turtles, for example, extensive keratinization may reduce cutaneous water exchange (Chilian, ’76) and potentially curb transmission of ice and INA. Willard et al. (2000) suggested that C. picta skin is both heavily keratinized and rich in lipid deposits, perhaps accounting for the profound inoculation resistance exhibited by this species. Other investigators have found that hatchling C. picta maintain concentrations of lipids in the skin (though not in carcass) throughout winter, but failed to link lipid density with the distinct seasonal/ontogenetic development of inoculation resistance (Fig. 8). Although these results do not preclude an important role for skin lipid in inoculation resistance, they seem contrary to the hypothesis that fat deposition accounts for the observed seasonal/maturational development of this character (Packard and Packard, 2003b). A particularly interesting finding in this line of research was that transmission properties of the integument varied anatomically, the skin within axillary and inguinal pouches posing less of a barrier to ice and INA than that on exposed appendages (Packard and Packard, ’95a).

Physiological regulation of inoculative freezing in hatchling turtles has received scant attention in the literature. In principle, organic osmolytes accumulated during winter acclimatization (e.g., Costanzo et al., 2000b, 2006) influence the process because INA activity is sensitive to the solute concentration in the nucleating environment (Zachariassen and Hammel, ’76). Some osmolytes apparently work cooperatively with specialized proteins to inhibit internal INA (Duman, 2002). Furthermore, in some organisms, AFPs not only protect against spontaneous freezing but also block inoculative freezing (DeVries, ’82; Zachariassen and Husby, ’82; Duman et al., ’91). It is believed that turtles lack such proteins, as their blood exhibits no thermal hysteresis (Storey et al., ’91; Costanzo et al., 2000b). However, it is possible that hatchlings harbor these proteins in concentrations too low to induce hysteresis (Ramlov et al., ’96) or solely in close association with the epidermis or other structures (Olsen et al., ’98; Duman, 2001).

Temporal and environmental influences

Inoculative freezing is influenced not only by an organism’s morphology and physiology, but also by factors determining duration of exposure to and intimacy with ice and INA. The process becomes increasingly efficacious as temperature falls below the tissue equilibrium freezing/melting point (Salt, ’63; Packard and Packard, ’93a; Packard, ’97; Packard et al., ’97b). For example, inoculation frequency for hatchling C. picta in
artificial nests increased more than 10-fold as temperature was reduced from −2.5 to −10.5°C (Fig. 9). The number of hatchlings inoculated inside thermostatic artificial nests increases over time, some individuals freezing many days after initial exposure to ice and INA (Packard and Packard, '93a; Packard et al., '99a). Apparently, freezing risk varies with seemingly small differences in exposure time, as the inoculation frequency of hatching C. picta held at −2.5°C in a frozen, loamy sand was 6% after 11 d (Packard et al., '97b), but 39% after 14 d (Packard et al., '99a).

Notwithstanding the importance of temperature and time, inoculative freezing is exquisitely sensitive to complex, indeterminable factors mediating intimacy between an organism and ice and INA in its immediate surroundings. Ambient moisture has a potent influence on freezing risk in diverse organisms (Salt, '63, '66; Layne et al., '90; Lundheim and Zachariassen, '93; Costanzo et al., '95a, '97, '98). For hatching turtles, inoculation frequencies of specimens in simulated hibernacula climb sharply with soil moisture content (Costanzo et al., '95b, '98, 2000a, 2001c; Baker et al., 2003). Moisture levels in natural hibernacula, such as C. picta nests, vary seasonally but often peak when temperatures are lowest, thus compounding freezing risk; consequently, mortality tends to be greater in winters that are both cold and wet (Costanzo et al., '98, 2004). The heightened inoculation susceptibility in wetter soils stems from a greater abundance of ice, and hence the higher probability of contacting crystals; moisture also facilitates transmission of soil INA to turtle tissues (Costanzo et al., 2000a, 2004). Laboratory studies with C. p. bellii (Costanzo et al., '98) and C. p. marginata (Costanzo et al., 2001c) cooled in native nesting soils showed that the relationship between soil moisture content and hatchling $T_c$ is logarithmic. The nature of this response permits inoculation under wet conditions at temperatures conducive to freezing survival (i.e., $\geq -4^\circ$C); at lower ambient moisture levels, the probability of inoculative freezing (and death due to uncontrolled ice formation) is greatly diminished (Fig. 10). Recall, however, that supercooling is ultimately constrained by the action of endogenous INA.

Moisture abundance in terrestrial hibernacula is governed by various environmental factors, including climatic and seasonal precipitation patterns, topography, and edaphics. Composition

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**Fig. 9.** Incidence of inoculative freezing in hatchling painted turtles (Chrysemys picta) as a function of ambient temperature. Hatchlings were cooled individually inside artificial nests consisting of a 473-mL glass jar containing frozen loamy sand and thus were exposed to ice and ice-nucleating agents (INA). Each point represents the percentage of turtles in the sample that froze. The number of turtles in each sample was as follows: North Dakota, $n = 32$ (Packard et al., '97b); Nebraska, $n = 30$ (Packard and Packard, '97) or $n = 23$ (Packard and Packard, 2003a); New Mexico, $n = 10$ (Packard et al., 2002); and Minnesota, $n = 18$ (Packard et al., '99a). The equation describing the line of best fit: $y = -7.56x + 6.75$ ($r^2 = 0.77$). Exposure to temperatures below the equilibrium melting/freezing point of turtle tissues lasted 9–11 d; exposure to the indicated target temperature lasted 1.5–9 d. Where reported, soil moisture was 16.4–25% (w/w) and water potential was −75 to −50 kPa.

**Fig. 10.** Temperatures of crystallization ($T_c$) of individual hatchling painted turtles (Chrysemys picta) cooled in contact with frozen nest soil as a function of soil moisture content and soil texture. Indiana turtles (C. p. marginata), hatched inside natural nests, were tested in a clayey soil collected from one of the nests at Mount Zion Mill Pond, Fulton Co., IN; hence, the lower limit of supercooling is constrained by ice-nucleating agents (INA). Nebraska turtles (C. p. bellii) hatched on vermiculite in the laboratory were tested in loamy sand collected from a nesting site in Garden Co., NE. All hatchlings were cooled in artificial nests in which the soil enveloping the turtle was frozen. (Adapted from Costanzo et al., 2001c.)
and texture (particle-size distribution) of soil strongly affect its drainage and moisture-retention characteristics. Soils comprised of large particulates drain readily, whereas soils containing organic matter or many fine particulates and colloids tend to hold moisture (Fig. 11). However, these entities also tend to adsorb water, lowering its activity and limiting ice formation (Forge and MacGuidwin, '92; Costanzo et al., '97). Moisture abundance in natural hibernacula has been examined in the context of inoculative freezing (Costanzo et al., '98, 2001c, 2004; Baker et al., 2003) and varies geographically as well as among hibernacula in the same general locale. Indeed, soil moisture content—and thus freezing risk—vary such that, during any given chilling episode, turtles in adjacent nests may be either frozen or supercooled (Costanzo et al., 2004). Differential freezing risk may account for gross differences in survival rates of hatchlings overwintering in neighboring hibernacula (Packard et al., '89, '98, 2003) and varies geographically as well as among hibernacula (Costanzo et al., '98, 2001c, 2004; Baker et al., '92; Costanzo et al., '97). Moisture abundance in natural hibernacula has been examined in the context of inoculative freezing (Costanzo et al., '98, 2001c, 2004; Baker et al., 2003) and varies geographically as well as among hibernacula in the same general locale. Indeed, soil moisture content—and thus freezing risk—vary such that, during any given chilling episode, turtles in adjacent nests may be either frozen or supercooled (Costanzo et al., 2004). Differential freezing risk may account for gross differences in survival rates of hatchlings overwintering in neighboring hibernacula (Packard et al., '89, '97a; DePari, '96; Packard and Packard, '97).

Findings of several laboratory studies underscore the importance of soil texture as an environmental determinant of freezing risk. Inoculation resistance in hatchling C. picta cooled in a matrix of native sandy soil was markedly improved after adding a small quantity of either clay or organic matter (peat) to the soil (Fig. 11). Packard and Packard ('97) found that inoculation frequencies of hatchling C. picta chilled to −7°C in artificial nest hibernacula were markedly higher with “loamy sand” (80%) as compared with “clayey soil” (33%), even though water potential of the two substrata was similar. In other studies of hatchling C. picta, the temperature at which half of an experimental group was inoculated was −6.5°C if the substratum was “loamy sand” (Packard et al., '97b), but was −9°C if turtles were exposed to “clayey soil” (Packard and Packard, '93a). Studies by Costanzo et al. (2001c, 2004) revealed that marked variation in inoculation resistance of C. picta hatchlings from different populations was due to differences in characteristics of their respective indigenous soils (Fig. 10).

The contention that soil composition influences inoculation susceptibility, and hence winter survival, of hatchling turtles draws support from field studies. Analyzing the winter mortality data reported by Packard ('97) and Packard et al. ('97a) for hatchling C. picta, Costanzo et al. ('98) noted that the survival of hatchlings occupying nests constructed in “loamy sand” exceeded that of turtles hibernating in “fine sand” in two consecutive years: 1994–1995 (94% vs. 65%) and 1995–1996 (90% vs. 36%). Because the soils in this area contain little clay or organic matter, indigenous turtles may freeze more readily than turtles endemic to areas with different soil types, other factors being equal (Costanzo et al., 2004).

Although the relative importance of soil characteristics to survivorship in field studies is confounded by variation in hibernaculum temperature, these field observations, together with available laboratory data, suggest that winter survival rates are linked to edaphics on both local and regional scales. This situation raises interesting questions about the maternal role of choosing the nest site. Selecting a clayey soil over a sandy one is not only beneficial to developing embryos (Legler, '54; Ratterman and Ackerman, '89), but...
in principal also promotes freeze avoidance. Perhaps fortuitously, in some populations, *C. picta* emerge in fall from nests constructed in sand, but overwinter in nests constructed in organic or loamy soils (DePari, '96).

Investigations of inoculative freezing in hatchling turtles most commonly focus on ice, the supreme nucleator. Nevertheless, various INA, including both inorganic and organic entities, naturally occur in the winter microenvironment and can also trigger freezing. Two studies have examined the inoculation susceptibility of *C. picta* by cooling hatchlings in a matrix of nesting soil that contained INA but no ice (Costanzo et al., '98, 2000a). The INA sharply reduced supercooling in some turtles. Moisten the soil slightly induced inoculation in a higher percentage of the sample, apparently by causing more soil to become lodged in axillary and inguinal pockets and nuchal skin folds. Thus, environmental moisture not only provides a direct source of ice crystals, but also facilitates communio of soil INA with body surfaces.

The literature hints of an important, as yet unexplored, interaction between environmental INA and inoculation efficacy in hatchling turtles. Most studies of inoculative freezing have, for the sake of convenience, used specimens produced by hatching eggs in an incubation medium of (INA-free) vermiculite. However, hatchlings exposed to natural INA are substantially more susceptible to inoculation. For example, Packard and Janzen ('96) found that *C. picta* hatched inside natural nests incurred a 44% inoculation frequency when cooled to −4.6 °C inside artificial nests containing clayey soil. The impact of INA contamination is evident even in hatchlings produced from eggs laid in natural nests but subsequently hatched on vermiculite. Using hatching *C. picta* produced in this manner, the inoculation frequency for specimens kept in loamy sand for 7 d at −2 °C was 26% in one study (Packard and Packard, 2003b) and 57% in another (Willard et al., 2000). The propensity for inoculation at such a high temperature illustrates the precarious nature of supercooling as a freeze-avoidance strategy, even in species (such as *C. picta*) that have a well-developed capacity to resist inoculative freezing.

**Taxonomic and ecological associations**

Abundance, activity, and inoculation efficacy of various endogenous and exogenous INA are influential factors in the evolution of cold-hardiness strategies of ectothermic animals (Duman et al., '95; Lee et al., '95; Vernon and Vannier, 2002). As we earlier discussed, efficacy by which environmental ice and INA seed the freezing of body fluids depends on a given organism’s anatomical and morphological constitution, ambient temperature, and physico-chemical characteristics of the nucleating environment. Assuming that inoculation resistance is a heritable trait on which natural selection can act, one might suppose that freeze avoidance varies among species, being particularly well developed in freeze-avoiding terrestrial hibernators. In testing this idea, Costanzo et al. (2001b) compared inoculative freezing resistance among hatchlings of eight taxa of terrestrial and aquatic turtles collected from both northern and southern locales. Using standardized trials, in which hatchlings were cooled in a matrix of frozen soil, mean $T_c$ values ranged from −0.8 to −13.6 °C and tended to be lower among terrestrial hibernators. Other studies, which examined inoculation resistance in individual species, including *C. picta* (Packard and Packard, '93a; Costanzo et al., '98, 2000a), *C. serpentina* (Packard and Packard, '90; Packard et al., '93), *G. geographica* (Baker et al., 2003), *E. blandingii* (Packard et al., 2000; Dinkelacker et al., 2004), and *T. scripta* (Packard et al., '97c), generally support the maxim that this trait is better developed in terrestrial hibernators.

The foregoing conclusion notwithstanding, an especially poor inoculation resistance is found in some terrestrial hibernators whose primary cold-hardiness strategy seems to be freeze tolerance. Lacking endogenous INA or specialized ice-nucleating proteins, inoculation by environmental ice or INA ensures that freezing commences at high temperatures and that ice growth is controlled and proceeds slowly, a condition requisite to freezing survival (Lee and Costanzo, '98). This system would benefit *T. ornata* (Costanzo et al., 2001b) and *M. terrapin* (Baker et al., 2006), freeze-tolerant species that commonly overwinter in sandy soil, potentially within reach of frost. Hatchling *E. blandingii* probably also belong to this group, as they tolerate somatic freezing (Packard et al., '99b; Dinkelacker et al., 2004; Costanzo et al., 2006) and poorly resist inoculative freezing (Packard et al., 2000; Dinkelacker et al., 2004). The case with *G. geographica* is sharply contrasting. These hatchlings hibernate terrestrially but are freeze intolerant; they exhibit an extraordinary ability to resist inoculative freezing (Baker et al., 2003).
Although inoculation resistance varies taxonomically, it remains equivocal as to whether this trait varies among populations of conspecifics. One study found marked differences in inoculation resistance in *C. p. bellii* from Nebraska and *C. p. marginata* from Indiana; however, the disparity could be explained by characteristics of the soil indigenous to each locale, rather than phenotypic variation (Costanzo et al., 2001c). Another study indicated that resistance exhibited by Nebraskan *C. p. bellii* far exceeded that of Arkansan *C. p. dorsalis*, a primarily southern subspecies (Costanzo et al., 2001b); however, this finding remains tentative because the sample contained relatively few hatchlings. Directly comparing results from different studies is not appropriate unless identical experimental protocols were used. That caveat notwithstanding, the literature suggests that inoculation resistance in *C. picta* varies little among populations in Nebraska (Packard and Packard, '93a,b, '95a), Minnesota (Packard et al., '99a), North Dakota (Packard et al., '97b), Illinois (Packard and Janzen, '96), and even New Mexico, near the southern limit of the species’ distribution (Packard et al., 2002).

Referencing findings for the New Mexico population, Packard and Packard (2003c) argued that inoculation resistance (and profound supercooling capacity) are not maintained by selection for cold hardiness because these traits would have no value in such a mild environment. However, climatological records from a U.S. National Weather Service station at Alamogordo, NM, ~200 km SSW of the source of the animals (Soccorro County), indicate that frost descends in the soil column to 51 cm, and certainly would penetrate turtle nests. Furthermore, contrary to the claim (Packard and Packard, 2003c) that this population was derived from ancestors that had never confronted cold stress, these turtles apparently are descended from northern populations (Starkey et al., 2003); thus, their cold-hardiness traits likely predate glacial expansion (Holman and Andrews, '94). Carefully controlled studies are needed to examine populational variation in this important trait, especially in terrestrial hibernators other than *C. picta*.

What is the basis for taxonomic variation in inoculation resistance? If skin is a primary barrier to ice and INA transmission, then, all else being equal, species (and individuals) having more exposed skin should be at greater risk of freezing through contact inoculation. This argument is also germane to water loss, which, in hibernating turtles, occurs primarily via transcutaneous routes (Bentley and Schmidt-Nielsen, '66). Indeed, Costanzo et al. (2001b) found that the rate of water loss was an excellent predictor of inoculation resistance among several taxa of terrestrial and aquatic hibernators, data from the exceptional *C. picta* excluded. This finding implies that both processes are mediated by the same morphological and/or physiological characters; it also explains why inoculation resistance is greater among terrestrial hibernators. Although the authors did not make direct measurements of cutaneous surface area, they suggested that the extensive plastron found in hatchlings of species that overwinter terrestrially limits exposure of the skin and mucous membranes of the cloaca to ice and INA.

Taxonomic variation in inoculation resistance may also derive from differences in the qualities of skin that influence its barrier properties. Willard et al. (2000) found a discrete lipid layer in the epidermis of hatchling *C. picta*, but no such layer in skin of *T. scripta, E. blandingii* and *C. serpentina*, species that exhibit poor resistance. A recent study (J. Costanzo and J. Jack, unpublished data) determined that inoculation resistance and skin lipid density were positively correlated ($r^2 = 0.62; P = 0.035$) in hatchlings of seven taxa of North American turtles, affirming the general importance of lipids in inoculation resistance (Fig. 12). Lipid density could underlie the strong link between inoculation resistance and dehydration resistance in hatchling turtles (Costanzo et al., 2001b).

How any hatchling could avoid freezing while overwintering in intimate contact ice and INA, if indeed they do, remains something of an enigma. One possible answer is that, in nature, transmission of ice and INA to the body fluids is imperfectly efficient. This notion is supported by the skewed distributions of $T_c$ values sometimes obtained in inoculation resistance trials (Costanzo et al., '98, 2000a). Another possibility is that turtles are to some extent buffered from ice and INA by eggshells or other debris within the nest cavity. Behavioral avoidance of these agents may also be important. Given that limb movements apparently permit soil to infiltrate the axillary and inguinal pockets and thereby contact skin that is susceptible to transmitting ice and INA (Packard and Packard, '97), a hatchling might limit its contact with these agents simply by retracting its head and limbs within the shell (Packard and Packard, '95a). Forming a tight cluster with one’s siblings (e.g., Breitenbach et al., '84; Tucker, '97; Andreas

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and Paul, '98) would further reduce exposure, particularly for individuals in the group’s center (Costanzo et al., 2000a, 2001c, 2004; Nagle et al., 2000).

The hypothesis that winter mortality depends on such factors as location within the hibernaculum poses some interesting questions about certain life-history traits of northern turtles. If one assumes the primary cause of death is freezing initiated by contact with ice and INA, and that a hatchling gains the greatest protection by being shielded by its siblings, then, all else being equal, a relatively large clutch should have a survival advantage. Costanzo et al. (2004) found that C. picta nests containing at least nine hatchlings had markedly higher survival and lower incidence of total winterkill compared with nests with fewer siblings. Laboratory experiments also support the idea that a hatchling’s location within the hibernaculum and proximity to siblings moderates its exposure to ice and INA, and hence survival of chilling episodes (Fig. 13). Additional research is needed to test these relationships rigorously, but the concept is interesting. The hypothesis argues for a strong directional selection for large clutches in turtles living at high latitudes and in cold habitats, and, in fact, northern populations of turtles do produce relatively large clutches, purportedly to offset a higher mortality associated with life in an extreme environment (Tinkle, '61; Gemmell, '70; Moll, '73; Iverson et al., '93; Iverson and Smith, '93). This “protected siblings” hypothesis suggests a mechanism on which such selection might operate, at least for freeze-avoiding, terrestrial hibernators. We note, however, that it does not preclude the possibility that large clutches also are beneficial, albeit through some other mechanism, in the more northern populations of species whose hatchlings hibernate aquatically.

Challenges imposed by the harsh winter environment may shape other life-history traits, such as propagule size. Contrary to the paradigm that larger individuals have a survival advantage, ice-nucleation theory predicts that a small body should favor survival in freeze-avoiding species. Ice nucleation occurs once sufficient water molecules have organized to form an ice embryo (Rasmussen and MacKenzie, '73) and, because the probability of embryo formation increases with fluid volume (Bigg, '53; Langham and Mason, '58; Vali, '95), small organisms tend to supercool more extensively than large ones. Furthermore, small animals, by virtue of their limited body surface, are less likely to incur inoculative freezing (Costanzo et al., 2001b).

Theory aside, the “smaller is better” concept advanced by Costanzo et al. (2004) has not as yet received rigorous empirical testing. One
laboratory study determined that incidence of (apparent) freezing mortality was in fact lower in lighter (smaller?) hatchling C. picta (Costanzo et al., 2001c); similar results have been reported for invertebrates (Holmstrup et al., 2007). On the other hand, in a Michigan field study of the same species, Nagle et al. (2000) found no association between mean body size and the proportion of hatchlings within individual nests surviving winter. However, this analysis included data from only eight nests in a single winter, and the authors used body mass, a labile metric, as proxy for body size for both live and dead turtles. A large-scale field study would be needed to thoroughly address this question, but some ancillary evidence suggests that such an effort may prove rewarding. Some northern turtles potentially can lay eggs larger than those actually produced, so selection apparently favors production of hatchlings of smaller than maximal size (Tucker, 2000b). Costanzo et al. (2004) conjectured that size-specific freezing risk could partly explain why C. picta populations in severely cold regions tend to produce relatively small offspring. Annual reproductive output of C. picta is more or less constant (relative to female body size) among populations at all latitudes and is partitioned within clutches via a trade-off between offspring number and size (Iverson, ’92; Iverson and Smith, ’93). Thus, any female that produces relatively small hatchlings also produces many hatchlings, this combination compounding the increase in her fitness. The possibility that the winter environment is a selective force shaping the life-history traits of turtles merits further consideration.

**Freeze tolerance**

Natural freeze tolerance is a complex, multifaceted adaptation derived from a coordinated suite of responses to the formation and melting of ice in tissues. It has evolved independently many times in diverse organisms. Long known in arthropods and other invertebrates, it was first recognized as a winter survival strategy among vertebrates only relatively recently (Schmid, ’82). These seminal investigations focused on woodland frogs, but it was soon discovered that certain reptiles also tolerated tissue freezing (Costanzo et al., ’88; Storey et al., ’88). Currently, freeze tolerance is known in anuran and urodele amphibians, both suborders of squamates, and turtles (Ramlov, 2000; Baker et al., 2004; Storey, 2006). Amongst hatchling turtles, most work has focused on C. picta; evidence for freeze tolerance in this species abounds (Storey et al., ’88; Paukstis et al., ’89; Claussen and Zani, ’91; Rubinsky et al., ’94; Costanzo et al., ’95b; Packard et al., ’99b; Hemmings and Storey, 2000; Willard et al., 2000; Packard and Packard, 2004).

In common usage, freeze tolerance denotes an organism’s ability to survive the freezing and thawing of a biologically significant quantity of body water under thermal and temporal regimes relevant to the organism’s life history (Baust, ’91). Unfortunately, the literature is fraught with reports that have failed to distinguish responses that are adaptive in the sense of evolutionary process (e.g., Bennett, ’97) from those of recovery from superficial injury (Storey and Storey, ’96). Indeed, some reports of freeze tolerance in invertebrates (Paukstis et al., ’96; Ansart et al., 2001; Cook, 2004), amphibians (Pasanen and Karhapää, ’97; Croes and Thomas, 2000; Steiner et al., 2000), and reptiles (Claussen et al., ’90; Andersson and Johansson, 2001; Burke et al., 2002) are based on experiments in which subjects have survived a measure of internal ice formation, but the tolerance is limited to superficial freezing over very brief exposures to relatively high temperatures. Compounding this problem, some authors have endeavored to catalogue various degrees of cold hardiness, including subcategories of freeze tolerance (Baile, ’93, ’96; Sinclair, ’99; Nedved, 2000; Vernon and Vannier, 2002; Voiturron et al., 2003; Hawes et al., 2006). These constructs, which have generated a litany of confounding idioms, including the oxymoron “partial freeze tolerance,” have done little to advance our understanding of freeze tolerance as a natural survival adaptation. Some authors hold in high regard the ability to revive after attaining an equilibrium ice content, irrespective of temperature (Sinclair, ’99; Voiturron et al., 2003); this criterion also lacks relevance to life history. Others have suggested recovery after 24 h of freezing at −2.5°C as the minimum basis for designating freeze tolerance in ectothermic vertebrates (Storey and Storey, ’92). These conditions, which generally result in the freezing of >50% of the body water (Claussen and Costanzo, ’90), undoubtedly are lethal to organisms lacking specific adaptations to somatic freezing. Adopting this criterion may provide an objective, discriminating test that can be applied broadly across taxa, but nevertheless fails to consider life-history variation amongst cold-hardy species. In our view, it is important to consider the demonstrated
capacity for tolerating somatic freezing in both physiological and ecological contexts. We recognize, however, the current paucity of ecological information for all but a few species.

Freeze tolerance is usually regarded as a winter survival strategy, but in temperate locales some organisms also are at risk of freezing early and late in the activity season. Instances of mass mortality of reptiles have been attributed to unseasonable frosts occurring in autumn and spring (Spellerberg, '76; Gregory, '82). Freeze tolerance presumably protects organisms from this hazard and conceivably can extend behavioral activity into the cooler months. For example, in the northern part of its range, T. carolina sometimes remains abroad until early November, well after the first autumnal frost (Claussen et al., '91), perhaps owing to its tolerance to somatic freezing (Costanzo and Clausen, '90; Costanzo et al., '93; Storey et al., '93). Similarly, the garter snake (Thamnophis sirtalis) encounters frost during spring and fall owing to its habit of emerging from hibernation early and retiring late (Vincent and Secoy, '78). Its ability to survive the freezing of at least 35% of its body water for at least 48 h (Costanzo et al., '88) is more important at these times than during winter, as this species usually hibernates in thermally buffered sites (Costanzo, '86; Lutterschmidt et al., 2006). A tolerance for somatic freezing, even if limited, could similarly protect hatchling turtles that encounter frost while abroad (Packard et al., 2000).

Organisms whose overwintering habitats vary with developmental stage commonly exhibit an ontogenetic shift in cold-hardiness strategy (Bouchard et al., 2006; Rinehart et al., 2006). It is possible for freeze tolerance to be expressed in neonatal turtles but lacking in older individuals (or conversely), although no such case has been discovered. Hatchling and adult box turtles (T. carolina and T. ornata) are freeze-tolerant, as both life stages overwinter terrestrially, potentially within the frost zone (Costanzo and Clausen, '90; Claussen et al., '91; Costanzo et al., '95b; Converse et al., 2002; Bernstein and Black, 2005). Adult mud turtles (K. flavescens) pass the winter deep in the soil column (Tuma, '93, 2006) and are intolerant of freezing, as are hatchlings (Costanzo et al., '95b). Freeze tolerance is well developed in hatchling C. picta (Storey et al., '88; Willard et al., 2000; Packard and Packard, 2004) and, although the adults can survive brief freezing of at least 15% of their body water (Johnson, '90; Claussen and Zani, '91; Claussen and Kim, '93), it is unclear whether this reduced ability is simply vestigial or if it enhances survival under certain circumstances. Adult C. picta usually hibernate aquatically and, thus, are buffered from extreme cold (Ultsch, '89, 2006); however, they become exposed to frost at least occasionally (Christiansen and Bickham, '89; St. Clair and Gregory, '90).

**Methodological considerations**

Laboratory studies must be carefully designed if they are to properly assess an organism's ability to tolerate freezing/thawing under ecologically relevant conditions. It is essential to control ice nucleation and to ensure that freezing proceeds slowly; spontaneous nucleation of deeply supercooled tissues is deleterious, even in profoundly freeze-tolerant species, because it promotes (lethal) intracellular freezing and accelerates ice accumulation, leaving cells insufficient time to adapt to volume change and hastening circulatory arrest (Storey and Storey, '92). For example, in the wood frog, rapid freezing is injurious (Costanzo et al., '91a) because, among other things, it hampers water flux and cryoprotectant mobilization (Costanzo et al., '91b, '92). It is also important to thaw the specimens slowly, to hold them at appropriate temperatures, and to allow adequate time for them to revive before assessing survival.

In practice, ice nucleation is readily induced by bringing ice or frozen substratum into contact with the wetted surface of a specimen that has been chilled slightly below its equilibrium freezing/melting point (Attaway et al., '98). Once freezing begins, the turtle can be slowly cooled to the prescribed temperature. This is easily accomplished by immersing the vessel containing the turtle in a refrigerated bath. It is important to hold the animal at the target temperature long enough such that ice content attains equilibrium; however, investigators should choose an exposure duration that is sensible in the context of the species' ecology. Finally, the specimen is thawed by permitting it to gradually warm to 0–4°C.

Survival criteria used in studies of freeze tolerance have not been standardized. Commonly, viability is inferred from the subject's physical appearance, passive behaviors, and response to mechanical stimulation. Although convenient, such endpoints are inherently subjective and could lead to bias. Costanzo et al. (2006) reported that plasma lactate dehydrogenase (LDH) activity was a reasonably objective marker of cryoinjury in hatchling turtles. The premise of this assay is that...
freezing perturbs cell membranes, permitting cytosolic contents to leak into the interstitium; thus, cryoinjury can be indexed by circulating levels of this easily measured enzyme.

Another concern regarding survival assessments is that few investigators monitor the post-trial vitality of frozen/thawed hatchlings for very long. Evaluations made shortly after freezing/thawing do not necessarily reveal injury or dysfunction manifested later in life (Baust and Rojas, '85; Ansart et al., 2002; Layne and Peffer, 2006). On the other hand, freeze tolerance is underestimated or entirely overlooked if survival is assessed before animals have fully recovered. Indeed, although some authors have reported heavy freezing mortality in hatchlings they examined only ~24 h after thawing (Packard et al., '97c, '99b; Packard and Packard, 2003a), several studies indicate that recovery requires from several days to perhaps a week, depending on the severity of the stress (Costanzo et al., 2004, 2006; Baker et al., 2006).

Because investigators do not always follow appropriate experimental practices, discretion should be used when interpreting results of freeze-tolerance trials.

**Limits of freeze tolerance**

The severity of freezing/thawing stress—and potential for cryoinjury and death—varies with factors such as minimum temperature, exposure duration, and rates of cooling and warming. Because the outcome of a freezing/thawing trial depends, in part, on the experimental conditions, determining an organism’s capacity for freeze tolerance is not a simple matter. Furthermore, freeze tolerance can be considered both in terms of minimum tolerable temperature, which has implications for the amount of ice being formed and associated cellular dehydration and osmotic/ionic perturbation tolerated, as well as the maximum duration, which reflects tolerance of additional stresses such as energy depletion and hypoxia tolerance.

Generally, freeze-tolerant animals can survive the freezing of up to two-thirds of their body water; formation of additional ice causes excessive cell dehydration and irreversible damage to cell membranes and organelles (Storey and Storey, '88). Because ice content increases as an organism cools, the survival limit can be associated with a critical minimum temperature (Claussen and Costanzo, '90), although this value may vary considerably with the physiological state and the developmental stage. Critical temperatures have not been defined for hatchling turtles, but seemingly are near −4°C. Tolerable ice contents of 40–55% of total body water have been measured in hatchling emydids frozen at an equilibrium temperature of −2.5°C (Storey et al., '88; Churchill and Storey, '92a,b).

As a general rule, freezing mortality increases with time spent in the frozen state (e.g., Sømme, '96; Ansart and Vernon, 2003). Although this principle has been amply documented in hatchling turtles (Churchill and Storey, '92a,b; Attaway et al., '98), physiological factors governing freeze endurance are not well known. Some evidence suggests that lactate accumulation and/or energy depletion are involved. As ice propagates within the body, cardiac activity diminishes and ultimately ceases (Rubinsky et al., '94), rendering tissues ischemic and reliant on anaerobic glycolysis to meet metabolic demands. Isolation of cells from nutrient delivery and waste management systems may contribute to their mortality (see Belkin, '63). Some authors have speculated that accumulated lactate, the main end-product of glycolysis, contributes to freezing mortality (Packard and Packard, 2004). Support for this idea comes from the recent finding that blood lactate and injury from experimental freezing were correlated among the hatchlings of various species (Costanzo et al., 2006). Time-dependent mortality may also reflect depletion of the metabolic fuels necessary to meet cellular demands for ATP (Layne et al., '98). Yet another explanation is that mortality coincides with onset of physical damage due to recrystallization, the gradual enlargement of small, unstable ice crystals at the expense of others (Knight and Duman, '86). Relatively little study has been devoted to defining limits of freeze endurance. Hatchling *C. picta* reportedly can survive freezing for 6–7 d at −2°C (Willard et al., 2000; Packard and Packard, 2003b) and at least 11 d at −2.5°C (Churchill and Storey, '92a). Several hatchling *C. picta* believed to be frozen recovered after spending 1 month inside an explanted natural nest kept at −2.5°C inside a laboratory incubator (Costanzo et al., 2001c).

**Adaptive mechanisms of freeze tolerance**

To survive even a single, mild freezing episode, organisms must withstand myriad stresses simultaneously manifested at multiple levels of biological organization. Such stresses derive from the...
formation and melting of ice in extracellular compartments, osmotic withdrawal of water from cells, and attendant osmotic and ionic effects on macromolecules, membranes, and cell homeostasis (Steponkus, '84; Storey and Storey, '88; Mazur, 2004). As the extracellular water freezes, a growing osmotic tension withdraws cytoplasmic water, thereby causing cells to shrink. The resulting hyperconcentration of solutes, together with the release of lysosomal proteases, can denature intracellular proteins.

Freezing impacts the physical state of lipids and can alter membrane fluidity and composition, impairing various membrane-mediated processes, such as transport and metabolism (Steponkus, '84; Bischof et al., 2002). As tissues freeze, the expanding ice front can force cells apart and damage capillaries. Mechanical and osmotic stresses can combine to irreversibly disrupt tissue architecture (Muldrew et al., 2004) and this result, if not directly injurious, may ultimately cause ischemic necrosis or apoptotic destruction of tissues (Hoffman and Bischof, 2005).

At the organ system level, pervasion of ice throughout the body eventually arrests breathing and cardiac function, rendering tissues ischemic and hypoxic (Churchill and Storey, '91, '92a; Packard and Packard, 2004). Consequently, energy stores are depleted and oxidative stress can occur when oxygen levels are restored on thawing; membrane damage resulting from oxidative stress is a potentially significant cause of freezing-related injury (Murphy, '83). Nervous tissues are particularly sensitive to freezing stress (Cameron, '30) and, accordingly, cryoinjury manifested as neurobehavioral dysfunction (e.g., lethargy, impassiveness to tactile stimulation, absence of the righting response) varies with severity of the freezing exposure (Costanzo et al., '95a; Attaway et al., '98; Burke et al., 2002; Dinkelacker et al., 2004).

Freeze-tolerant organisms employ a suite of coordinated molecular and physiological responses that minimize injury caused directly or indirectly by the formation and melting of ice (for reviews, see Storey and Storey, '88; Storey, '90a). Some of these protective mechanisms probably originated from fundamental responses to osmotic and hypoxic stress. For many species, adaptive strategies of freeze tolerance include control of the ice-nucleation event, management of the redistribution of water and solutes, hypometabolism, upregulation of antioxidant defense systems, and expression of genes involved in homeostasis and somatic repair. In addition, freeze-tolerant organisms commonly accumulate cryoprotectants, organic osmolytes that reduce ice formation and the attendant cell dehydration, and also preserve the structural integrity of proteins, membranes, and other cell structures (Storey, '97; Mazur, 2004; Yancey, 2005). Principles of natural freeze tolerance are reasonably well known, especially as they pertain to invertebrates and anurans. Freeze tolerance is also well developed in certain reptiles, but the underlying molecular and physiological mechanisms remain incompletely understood (Costanzo et al., 2006; Storey, 2006).

Seasonal variation in freeze tolerance

Capacity for freeze tolerance varies in association with cold hardening or winter acclimatization in many temperate ectotherms (Aarset, '82; Murphy, '83; Lee, '91), including hatchling turtles. Field investigations of *C. p. bellii* demonstrated that, in two consecutive years of study, freeze tolerance was weakly expressed in late summer, but had improved markedly during winter and persisted at least into April (Costanzo et al., 2004). Similarly, Churchill and Storey ('92a) reported that hatching *C. p. marginata*, collected from natural nests at winter's end, were substantially more freeze tolerant than a November cohort; thus, cold hardening is required for the full expression of freeze tolerance. Laboratory experiments also suggest that a period of cold acclimation improves freeze tolerance in hatching *C. picta* (Packard and Packard, 2003b). Factors regulating this change are as yet unknown, but may relate to metabolic and osmoregulatory shifts accompanying seasonal cold hardening. For example, enhanced freeze tolerance in winter animals could derive from accumulation of urea (Costanzo et al., 2000b, 2006), an efficacious cryoprotectant in some species (Costanzo and Lee, 2005). Seasonal variation in freeze tolerance could also reflect shifts in plasma membrane composition, structure, and function that render this sensitive structure more tolerant to freezing-induced stresses (Orvar et al., 2000). Membrane remodeling probably occurs in hatching turtles during preparation for winter. Notably, an increase in the membrane cholesterol level, which is known to stabilize membranes during cooling (Drobnis et al., '93), has been observed in hatching *C. picta* undergoing cold acclimation (Fig. 14).

Ice-nucleating agents

Some freeze-tolerant organisms synthesize INA that initiate and control the freezing process,
guarding against damage from rapid, uncontrolled ice growth in deeply supercooled tissues (Storey and Storey, '88; Duman et al., '91; Lee and Costanzo, '98). Known classes of endogenously produced INA include inorganic crystals and the more common ice-nucleating proteins. An early report of ice-nucleating activity in the range of −7 to −8°C in the blood of hatchling C. picta seemed to indicate that endogenous INA could play a role in turtle cold hardiness (Storey et al., '91). However, subsequent results argue that the activity was an artifact of sample contamination with exogenous INA (Costanzo et al., 2000b). In the latter study, C. picta blood exhibited ice-nucleating activity if turtles were hatched and reared on nesting soil containing native INA, but not if they were reared on vermiculite, a substratum lacking soluble INA. The ability of vermiculite-reared C. picta to supercool to nearly −20°C (Costanzo et al., 2000a) is perhaps the best evidence that endogenous INA are not used to regulate ice nucleation. Rather, inoculation by environmental ice and INA serves this critical function (Costanzo et al., '95b).

Antifreeze agents

AFPs and glycoproteins, more aptly termed “ice-structuring proteins” (Clarke et al., 2002), are best known for their role in arresting the growth of ice and preventing wholesale tissue freezing. However, in some freeze-tolerant organisms, these specialized agents help prevent recrystallization, a thermodynamic phenomenon in which some ice crystals gradually enlarge within tissues at the expense of others (Knight and Duman, '86; Loomis, '91; Tursman et al., '94; Ramlov et al., '96; Duman et al., 2004). If unchecked, growing crystals could irreparably damage tissue architecture and disrupt intercellular communication systems. Turtle blood lacks thermal hysteresis (Storey et al., '91; Costanzo et al., 2000b), suggesting a lack of AFPs, but whether or not these agents are actually present (and effective in preventing recrystallization) in concentrations too low to be detected is unknown.

Bound water

The equilibrium freezing/melting point of water is lowered not only by the colligative effects of dissolved solutes but also by the hydration of macromolecules, membranes, and subcellular structures. Water molecules proximal to hydrophylic surfaces are subject to attractive forces that greatly reduce their potential energy (Wolfe et al., 2002). Known as “bound water,” this fraction has an exceedingly low freezing point and does not

Fig. 14. Cholesterol content in plasma membranes of erythrocytes and enterocytes of hatchling painted turtles (Chrysemys picta) during seasonal cold hardening. Eggs were collected from gravid females at a nesting area in Garden Co., NE, and incubated in the laboratory at ~29°C until hatching in August. Turtles were reared on vermiculite, kept in darkness, and progressively acclimated to 4°C; experiments were conducted on 15 October with 20°C-acclimated hatchlings (n = 9), 15 November with 15°C-acclimated hatchlings (n = 9), and 15 December with 4°C-acclimated hatchlings (n = 10). Erythrocytes and a 10-mm section of small intestine were harvested from euthanized turtles and randomly assigned to one of three separate pools, each containing tissue from 3–4 individuals. Membranes were isolated by homogenizing and sonicating samples in buffer (0.15 M NaCl, 3 mM MgCl2, and 0.01 M Tris-HCl, pH 7.2) and centrifuging (500 g, 5 min) the suspension. Membranes in the resultant suspension were collected by centrifugation (25,000 g, 15 min), washed in buffer containing 0.1 M choline chloride, and again centrifuged. The membrane pellet was used in spectrophotometric assays for cholesterol (No. 234-60, Diagnostic Chemicals Limited, Oxford, CN) and protein (Bio-Rad, Hercules, CA) assays. Means (±SE; n = 3) identified by different letters were statistically distinguishable (ANOVA; Student–Newman–Kuels, P < 0.05). (M. Polin and J. Costanzo, unpublished data.)
crystallize at the temperatures usually encountered in natural hibernacula; consequently, the quantity of bound water in tissues influences the amount of ice that forms at any given temperature (Murphy, '83; Claussen and Costanzo, '90). The bound water fraction can change seasonally in some temperate organisms (Storey et al., '81), an increase sometimes accompanying accumulation of micro- and macromolecules (Ring, '82; Issartel et al., 2006). Hatchling turtles accumulate osmolytes during cold acclimation (Costanzo et al., 2000b, 2006) and maintain large deposits of glycogen, a water-binding polymer (Rubinsky et al., '94; Hemmings and Storey, 2000), but whether or not bound water contributes significantly to freeze tolerance has not been tested directly.

**Water balance**

Many cold-hardy organisms dehydrate partially as they physiologically prepare for winter. This response can benefit freeze-tolerant species by concentrating osmolytes and by increasing the fraction of bound water in tissues (Ring and Tesar, '81; Storey et al., '81; Aarset, '82; Murphy, '83; Lee and Costanzo, '93; Hayward et al., 2007), both effects reducing the percentage of body water that freezes. Reducing the hydration of tissues also lowers ice content, thereby limiting mechanical damage to the microvasculature and other sensitive structures. In principle, freeze-tolerant turtles would benefit from partial dehydration, although the relationship between body water content and freeze-tolerance capacity has not been investigated. It is also possible that freeze tolerance is better developed in species that maintain relatively low hydration levels; however, although tissue water concentration does vary among taxa (Costanzo et al., 2001b, 2006; Dinkelacker et al., 2005b), Costanzo et al. (2006) found no support for the expected correlation.

**Ischemia and anoxia tolerance**

As a group, turtles exhibit a remarkable ability to survive oxygen lack (Lutz and Storey, '97; Bickler and Buck, 2007). This trait permits them to estivate or overwinter in habitats that otherwise would be inhospitable or lethal (Ultsch, '89; Crocker et al., '99; Jackson, 2002). Turtles are endowed with a host of mechanisms for coping with anoxia, including metabolic depression, efficacious management of oxidative stress, and tolerance of ionic and acid–base perturbations. They contend with lactacidosis by mobilizing calcium carbonate and magnesium carbonate from the skeleton (especially the shell), which buffer most excess protons, and by sequestering lactate in the shell (Jackson, 2000b). Hatchlings do not tolerate oxygen deprivation as well as adults, perhaps because their lactate buffering system is incompletely developed (Reese et al., 2004b; Dinkelacker et al., 2005a; Ultsch and Reese, 2008).

Anoxia tolerance is relevant to freeze tolerance because as ice propagates through the body, circulatory function slows and ultimately ceases (Claussen and Kim, '93; Costanzo et al., '93). This leaves tissues without oxygen, rich in lactate, and acidic (Churchill and Storey, '91, '92a, b; Packard and Packard, 2004). That freezing induces acute anoxic stress in hatchling turtles is evidenced by rapid accumulation of lactate in brains of *C. picta* and *T. scripta* (Hemmings and Storey, 2000).

Anoxia tolerance may be requisite to the evolutionary development of freeze tolerance (Storey et al., '88; Greenway and Storey, '99). Taxonomic variation in anoxia tolerance, which reportedly reflects differences in buffer reserves and metabolic rates (Reese et al., 2004b; Dinkelacker et al., 2005a, b), could explain differential capacities for freeze tolerance among hatchling turtles. Results of one study failed to support a strong association between these traits, leading the authors to conclude that, although anoxic stress is certainly relevant to freezing survival, it is not necessarily limiting (Dinkelacker et al., 2005b). Nevertheless, given that oxygen limitation is a principle determinant of thermal tolerance in ectothermic vertebrates (Pörtner, 2002), and that tissue ischemia underlies some of the important gene-expression responses to somatic freezing (Storey, 2006), anoxia tolerance appears to be a key exaptation in natural freeze tolerance.

**Antioxidant systems**

Oxidative stress occurs whenever production of ROS, such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH·), in the mitochondrial respiratory chain overtaxes the antioxidant defense mechanisms. Superoxide and hydrogen peroxide are not particularly reactive, but they can be readily converted into the harmful hydroxyl radical. Ischemia associated with somatic freezing can result in oxidative stress manifested when oxygen returns to the tissues after thawing (Storey, '96). Maintaining high constitutive levels of antioxidant enzyme activity...
is a defensive strategy employed by species prone to oxidative stress, although some organisms also (or instead) upregulate antioxidant defenses either in anticipation of, and/or in direct response to, increasing levels of ROS production (Hermes-Lima et al., '98; Hermes-Lima and Zenteno-Savin, 2002; Voituron et al., 2005b). In aquatic turtles, the antioxidant complement tracks seasons and generally decreases with body temperature, but remains relatively high in winter, apparently as an adaptation to submerged hibernation (Pérez-Pinzón and Rice, '95). Freezing and/or thawing-induced enhancement of antioxidant defenses, including increases in antioxidant enzyme activities, has been reported in freeze-tolerant vertebrates, including the wood frog (Joanisse and Storey, '96), garter snake (Hermes-Lima and Storey, '93), and common lizard (Voituron et al., 2005b).

Dinkelacker et al. (2005b) investigated responses of the antioxidant enzyme catalase (which combats oxidative stress by decomposing hydrogen peroxide) to freezing/thawing and anoxia exposure in hatchlings of seven turtle species, both freeze-tolerant and freeze-intolerant. All species exhibited high constitutive levels of the enzyme in liver, although the levels varied taxonomically. Although catalase activity increased during anoxia in most species, it increased with experimental freezing/thawing only in the poorly freeze-tolerant species. This outcome could mean that freeze-tolerant species maintain adequate constitutive levels of catalase, or that they tolerate high levels of free radical damage. Alternatively, perhaps other antioxidants are more important in the freezing response, or the experimental freezing regimen (2 d at −2.5°C) simply was too mild to cause significant ROS production in the freeze-tolerant species.

To help resolve the role of antioxidant defenses in reptilian freeze tolerance, antioxidant capacity and indices of oxidative damage in tissues of hatchling C. picta subjected to a 48-h bout of freezing (−2.5°C), followed by gradual thawing and 24 h of recovery were examined by Baker et al. (2007). Samples of plasma, brain, and liver were assayed for Trolox-equivalent antioxidant capacity, TBARS, markers of oxidatively damaged lipids, and protein carbonyls, markers of oxidatively damaged proteins. Finding an increase only in plasma TBARS, the authors concluded that freezing/thawing induced little stress. Hatchling C. picta, like other freeze-tolerant vertebrates (Hermes-Lima and Storey, '93; Joanisse and Storey, '96; Voituron et al., 2005b), apparently have a well-developed antioxidant defense system that minimizes oxidative damage stemming from freezing/thawing. However, whether or not oxidative damage contributes to cryoinjury of freeze-intolerant species remains to be determined.

Cryoprotectants

Certain organic osmolytes accumulated before, during, or perhaps after freezing substantially improve the tolerance of cells and tissues to freeze/thaw stresses (Storey, '97; Mazur, 2004; Yancey, 2005). These compounds, cryoprotectants, have multiple roles in freeze/thaw protection. When present in high concentrations, osmolytes, such as polyhydric alcohols and sugars, colligatively reduce ice formation and, if membrane permeable, cellular shrinkage. Some compounds, including certain amino acids and polyols, can function in much lower concentrations to stabilize membranes and macromolecules. Some serve as antioxidants or metabolic fuels, or increase the bound water fraction.

Although various freeze-tolerant invertebrates and amphibians accumulate cryoprotective solutes to high concentrations (e.g., 0.1–1 M or more), this seems not to be the case with reptiles, including hatchling turtles. In fact, although freezing usually triggers an osmolyte response in hatchling C. picta, glucose levels rise only modestly (Churchill and Storey, '92a) or not at all (Hemmings and Storey, '92a; Churchill and Storey, '92a; Costanzo et al., 2000b, 2004). It remains equivocal whether glucose is synthesized during freezing or during thawing. In a recent study, blood glucose levels in frozen/thawed hatchlings representing nine turtle taxa were 5- to 20-fold higher than in unfrozen counterparts. The finding that glucose accumulated in freeze-intolerant species as well as freeze-tolerant ones suggests that the mobilization is a fundamental response to stress, rather than an adaptation specific to freeze tolerance (Costanzo et al., 2006).

The aforementioned results pertain to turtles subjected to experimental freezing/thawing and should be interpreted cautiously. Few studies have examined the physiology of turtles hibernating in nature, although the available data do suggest they become hyperglycemic in winter (DePari, '88; Costanzo et al., 2004). Costanzo et al. (2004) found that plasma glucose in hatchling C. picta increased up to 15-fold in winter over levels prevailing in autumn, the onset of hyperglycemia coinciding with the advent of frost in the hibernaculum.
Glycemia tended to decrease as winter progressed, albeit remaining above autumn levels, and was higher in the colder of the two winters of record. Indeed, turtles encountering periods of chilling to subzero temperatures became profoundly hyperglycemic, with glucose concentrations in some individuals reaching 60 mmol L⁻¹. A strong, direct correlation between plasma glucose level and plasma osmolality attested that this metabolite is an important osmolyte in hatchling plasma osmolality.

Investigation of the putative roles of cryoprotectants in reptilian freeze tolerance has focused mainly on glucose and lactate. However, it is important to note that, in sufficient quantity, any permeable, compatible solute will coligatively moderate ice formation and limit cell shrinkage. Investigators have screened the blood and other tissues of freezing-exposed turtles for amino acids (Storey et al., ’88; Churchill and Storey, ’92a), sugars such as sorbitol, fructose, and mannose (Churchill and Storey, ’92a), and the metabolic end products alanine and succinate (Churchill and Storey, ’91), but found that these compounds do not rise with freezing or are present only in minute amounts. Apparently, glucose and lactate synthesis accounts for essentially all of the measured increment in osmolality (up to 77 mosmol kg⁻¹ in hatchling C. picta) associated with freezing (Costanzo et al., 2006).

Efforts to elucidate the role of cryoprotectants in reptilian freeze tolerance have been hampered by a singular focus on osmolyte accumulation in response to freezing. This mindset emanates from studies of freeze-tolerant frogs, which were found to mobilize large quantities of glucose and glycerol after freezing commenced, rather than during cold acclimation (Schmid, ’82; Storey and Storey, ’85). The anuran cryoprotectant system is unusual because most freeze-tolerant organisms accumulate osmolytes in anticipation of freezing (Storey and Storey, ’88; Lee, ’91). Only recently, however, was it reported that terrestrial hibernating, freeze-tolerant frogs accumulate a cryoprotectant, urea, in autumn and early winter (Layne and Jones, 2001; Costanzo and Lee, 2005). Whether or not reptiles also show an anticipatory response requires more study, but preliminary findings suggest that they do. Costanzo et al. (2000b) reported that seasonal development of cold hardiness in hatchling C. p. bellii was associated with an increase in plasma osmolality chiefly caused by accumulation of urea (to 80 mmol L⁻¹), the principle nitrogenous waste product in most chelonians. Subsequently, elevated plasma urea (30–50 mmol L⁻¹), accounting for ~10% of the total plasma osmotic potential, has been found in cold-acclimated hatchlings of several other species (Costanzo et al., 2006). Hatchlings do not necessarily remain hyperuremic throughout winter (Costanzo et al., 2004), but those that do stand to benefit from this solute’s cryoprotective properties. Indeed, urea accumulation could underlie the observed increase in freeze tolerance associated with winter acclimatization (Churchill and Storey, ’92a; Packard and Packard, 2003b; Costanzo et al., 2004). Additional research is needed to determine whether accumulated urea is perturbing, as little is known about the levels of trimethylamine oxide and other “counteracting” methylamines in turtle tissues.

Although hatchling turtles can accumulate organic osmolytes both before and during freezing/thawing, the importance of such compounds in freezing survival remains equivocal. On the one hand, organic solutes collectively account for up to one-third of total plasma osmotic pressure.
in frozen/thawed animals; without them, substantially more ice would form, mechanical stress and cellular dehydration would increase, and turtles would sooner and at higher temperatures attain a critical ice content (Costanzo et al., 2006). On the other hand, it is uncertain that lactate exhibits the most essential quality of any cryoprotective agent, and that of cytoprotectants in general: compatibility with cellular processes. Packard and Packard (2004) suggested that excessive lactate accumulation, failure of the buffering system, and attendant acid–base disturbance contributed to freezing mortality in hatchling turtles. Working with seven species, Dinkelacker et al. (2005b) found no clear relationship between freeze-tolerance capacity and blood lactate levels in frozen/thawed hatchlings. To the contrary, Costanzo et al. (2006) examined nine turtle taxa, finding lower rates of freezing survival in those with copious lactate, as well as a direct association between lactemia and cryoinjury among conspecific individuals. Disparity in experimental outcomes reported by Dinkelacker et al. (2005b) and Costanzo et al. (2006) probably reflects the comparatively colder and longer freezing exposure, which resulted in two-fold higher lactate concentrations, in the latter study. Unfortunately, those authors could not ascertain whether the link between hyperlactemia and freezing injury simply reflected differential lactate clearances among injured and healthy specimens, or whether it stemmed from adverse effects of the osmolyte (e.g., acidification) or the stress (e.g., hypoxia) triggering its accumulation. In any case, the available literature concurs that the hatchlings of freeze-tolerant species can recover from freezing/thawing without the need to accumulate large quantities of colligative cryoprotectants. This also is the case with a few freeze-tolerant insects (Salt, '61; Ring and Tesar, '81), most intertidal invertebrates (Murphy, '83; Ansart and Vernon, 2003), and reptiles, including adult turtles, snakes, and lizards.

**Gene and protein responses**

Innovations in molecular biology, such as cDNA library screening, DNA array screening, and real-time PCR, have greatly facilitated efforts to identify the myriad gene and protein responses supporting natural freeze tolerance (Storey, 2004a). In freeze-tolerant organisms, changes in protein and gene expression occur preparatory to winter or are triggered directly by the freezing event (Storey, '99, 2004b). Most of the relevant research on vertebrates has focused on freeze-tolerant frogs, but recent investigations have found in organs of hatchling _C. picta_ several classes of freezing-responsive genes, the products of which include antioxidant enzymes, inhibitors of serine protease, and iron-binding proteins (Storey, 2006). One major finding emanating from this line of research is that various genes responsive to anoxia or desiccation also respond to freeze/thaw stress, bolstering the view that freeze tolerance developed from fundamental mechanisms for coping with oxygen deprivation and cell volume regulation.

**Somatic correlates**

Relationships between somatic and life-history traits and cold-hardiness strategies of ectothermic animals are of potential interest to adaptational biologists, yet have received scant coverage in the literature. The ice nucleation theory predicts that as body size increases, supercooling becomes less effective as a cold-hardiness strategy. Thus, relatively large organisms may be predisposed to evolving a strategy of freeze tolerance rather than freeze avoidance. In addition, because a larger body will cool more slowly, ice growth in relatively large individuals tends to be more controlled and, overall, less body water will freeze before the animal rewarms (Claussen and Zani, '91; Voituron et al., 2005a). Body size bears significantly on freeze-tolerance capacity in some taxonomic groups, such as mollusks (Loomis, '95; Ansart and Vernon, 2003, 2004). Among hatchling turtles, cryoinjury tends to be less severe among larger individuals (Costanzo et al., 2006), this finding being consistent with the idea that freeze tolerance is more easily developed in larger species. Hatchlings of species that usually overwinter terrestrially tend to have larger supplies of yolk and storage lipids than species whose hatchlings hibernate aquatically (Congdon et al., '83c; Congdon and Gibbons, '85, '90; Rowe et al., '95; Nagle et al., '98; Costanzo et al., 2000b). Not only is residual yolk important to the energetics of aphasis, overwintering hatchlings, but it also could enhance their cold hardiness (Bleakney, '83). Freezing reportedly increases the vascularity and shrinks the internalized yolk sac, presumably as yolk materials are mobilized to other tissues (Hemmings and Storey, 2000). Costanzo et al. (2006) found no association between residual yolk mass and freeze-tolerance capacity among nine taxa of temperate North American turtles.
including both freeze-tolerant and freeze-intolerant ones. However, this result may not be definitive because little yolk remained when the turtles were tested, in late winter.

Maintaining a large glycogen reserve could contribute to freezing survival in hatchling turtles. Freeze-tolerant species have ample supplies of this compound, which, for example, may account for over one-half of the liver’s mass (Hemmings and Storey, 2000). Glycogen, by virtue of its water binding, helps reduce ice formation in tissues and also provides the freezing-mobilized solutes, glucose and lactate, putative cryoprotectants (Churchill and Storey, ‘91; Hemmings and Storey, 2000). Dinkelacker et al. (2005b) found that liver mass (normalized to body size) varied among hatchlings of seven turtle taxa, perhaps reflecting similar variation in glycogen reserves; however, these authors found no support for the expected relationship between liver mass and capacities for freeze tolerance or anoxia tolerance. That glycogen supply does not limit anoxia tolerance in hatchling turtles is further supported by the finding that 33–50% of the initial amount remains at the limit of survival (Reese et al., 2004b).

Taxonomic and ecological associations

Distinguishing degrees of freeze tolerance among turtle taxa is not a simple matter, partly because the thermal range for survival extends only to −4°C in even the most profoundly freeze-tolerant species. In addition, comparing literature reports from different laboratories is confounded by inconsistency in methodologies and criteria for assessing freeze tolerance. A few studies, in which investigators tested hatchlings using identical methods, attest that freeze tolerance varies considerably among species (Costanzo et al., ’95b, 2006; Packard et al., ’99b; Willard et al., 2000; Dinkelacker et al., 2005b). In a study of nine taxa of temperate North American turtles, viability of hatchlings subjected to experimental freezing (72 h at −3°C) ranged 0–100% and recovery rates varied among those surviving (Costanzo et al., 2006). Furthermore, the consensus of reports on individual species is that freeze tolerance is more fully developed in some taxa than in others. Hatchling C. picta, T. ornata, E. blandingii, and M. terrapin can recover from freezing to relatively low temperatures and for long periods (Storey et al., ’88; Dinkelacker et al., 2004; Baker et al., 2006; Costanzo et al., 2006). In contrast, hatchling T. scripta, G. geographic, and C. serpentina tolerate only moderate freezing exposures (Churchill and Storey, ’92b; Packard et al., ’93, ’99b; Costanzo et al., ’95b, 2006; Baker et al., 2003; Dinkelacker et al., 2005b); yet this limited capacity is distinct from that of other northern species (e.g., S. odoratus, K. flavescens, A. spinifera), which succumb to even mild freeze/thaw stress (Costanzo et al., ’95b, 2006).

Freeze tolerance has been examined in hatchlings from all four families (Chelydridae, Emydidae, Kinosternidae, Trionychidae) of freshwater turtles found in North America. Such studies have disproportionately focused on the Emydidae, a family of mostly Northern American pond turtles (Ultsch, ’89), although we suspect that freeze tolerance is not common in other family groups; for example, only C. serpentina (Chelydridae) tolerates even a modicum of freezing (Packard et al., ’93; Costanzo et al., ’95b, 2006; Dinkelacker et al., 2005b). No studies of freeze tolerance are available for any testudinid (tortoise), geoemydid (Asian pond turtle), sea turtle (chelonid or dermochelyid), or side-necked turtle (chelid, pelomedusid, or podocnemid), although all of these are subtropical or tropical in nesting distribution. Among emydids, freeze tolerance is found in both clades, occurring in at least two genera of the Emydidae (Eurasian and North American terrestrial forms) and in at least two genera of the Deirochelyinae (North American aquatic forms), suggesting that it is widespread in this family. Nevertheless, at least one northern emydid (G. geographic) is decidedly intolerant of freezing (Baker et al., 2003) and further research may identify others.

Various questions about phylogenetic aspects of the evolution of chelonian freeze tolerance remain unanswered because too few species and too few major groupings have been investigated. The northern distribution and life histories of certain turtles (e.g., E. orbicularis, T. horsfieldii) make them interesting candidates for freeze-tolerance testing. Unfortunately, many species may never be investigated owing to concerns regarding their conservation status. Clues to their freeze-tolerance status could be gathered through careful field observation. For example, Chase et al. (’89) noted that bog turtles (C. mühlenbergi) in Maryland overwintered successfully in close proximity to frozen mud and ice, questioning whether these turtles had a special physiological mechanism allowing them to survive in the frost zone. In Quebec, hatchling wood turtles (C. insculpta), monitored as they dispersed from a natal nest in late September, apparently became frozen during
an overnight frost, but before noon of the following day had thawed and revived, most having already resumed migration (A. Walde, personal communication).

Freeze tolerance generally is well developed in species known (or suspected) to hibernate in microenvironments where freezing is likely and poorly developed in species that overwinter in frost-free sites (Table 5). Terrestrial hibernators are not necessarily freeze tolerant, however. For example, hatchling *K. flavescens* do not tolerate freezing, but they commonly hibernate in the soil column beneath the nest, well below the reach of frost (Costanzo et al., ’95b). Hatchling *T. ornata* also overwinter beneath the natal nest, although they do not always evade frost (Doroff and Keith, ’90); this species tolerates somatic freezing (Costanzo et al., ’95b, 2006). Freeze tolerance in hatchling *M. terrapin*, which can successfully overwinter on cold, exposed estuarine beaches, is robust, but nevertheless inferior to that of more cold-adapted species (Baker et al., 2006). Although hatchling *C. picta* reportedly survive freezing at −2.5°C for at least 11 d (Churchill and Storey, ’92a), *M. terrapin*, which does not range as far north, apparently cannot.

There are some notable exceptions to the general association between hibernation habitat and ability to withstand freezing/thawing. For instance, although *G. geographica* sometimes encounters subzero temperatures while overwintering within the natal nest, they do not tolerate even mild freezing; instead, they are adept at freeze avoidance (Baker et al., 2003). Hatchling *C. serpentina*, which usually hibernate under water, can tolerate freezing under relatively mild conditions (Packard and Packard, ’90; Packard et al., ’93; Costanzo et al., ’95b, 2006; Dinkelacker et al., 2005b). Terrestrial overwintering of these hatchlings occurs at least occasionally (Bleakney, ’63; Obbard and Brooks, ’81a; Costanzo et al., ’95b), but whether they benefit from such limited

### TABLE 5. Summary of results of experimental tests of freeze tolerance in hatchlings of various taxa of North American turtles

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Hibernation habitat</th>
<th>Freeze-tolerance capacity</th>
<th>Equilibrium temperature tested (°C)</th>
<th>Duration tested (h)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apalone spinifera</em></td>
<td>Aquatic</td>
<td>Low</td>
<td>−3.0</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td><em>Chelydra serpentina</em></td>
<td>Aquatic</td>
<td>Low</td>
<td>−2.0 to −4.0</td>
<td>24–168</td>
<td>1–4</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>Natal nest</td>
<td>High</td>
<td>−2.0 to −4.0</td>
<td>24–226</td>
<td>1–10</td>
</tr>
<tr>
<td><em>C. p. marginata</em></td>
<td>Natal nest</td>
<td>High</td>
<td>−2.5 to −4.0</td>
<td>16–264</td>
<td>4, 9, 11</td>
</tr>
<tr>
<td><em>Emydoidea blandingii</em></td>
<td>Unknown; possibly terrestrial</td>
<td>High</td>
<td>−2.0 to −3.6</td>
<td>24–168</td>
<td>2–4, 12, 13</td>
</tr>
<tr>
<td><em>Graptemys geographica</em></td>
<td>Natal nest</td>
<td>Low</td>
<td>−2.5 to −3.0</td>
<td>24–72</td>
<td>3, 4, 14</td>
</tr>
<tr>
<td><em>Kinosternon flavescens</em></td>
<td>Below natal nest</td>
<td>Low</td>
<td>−2.6</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td><em>Malaclemys terrapin</em></td>
<td>Natal nest, other terrestrial, or aquatic</td>
<td>High</td>
<td>−2.5 to −3.5</td>
<td>26–288</td>
<td>4, 15</td>
</tr>
<tr>
<td><em>Sternotherus odoratus</em></td>
<td>Aquatic</td>
<td>Low</td>
<td>−3.0</td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td><em>Terrapene ornata</em></td>
<td>Below natal nest</td>
<td>High</td>
<td>−2.5 to −3.0</td>
<td>36–168</td>
<td>1, 3, 4</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Natal nest</td>
<td>Equivocal</td>
<td>−2.0 to −3.0</td>
<td>24–168</td>
<td>2–4, 7</td>
</tr>
</tbody>
</table>

Freeze-tolerance capacity was considered high if most experiments resulted in survival after freezing at ≥−2.5°C for 48 h. Survey included experiments in which turtles began freezing at ≥−2.0°C, were exposed to equilibrium temperatures between −2.0 and −4.0°C, and remained frozen ≥24 h. References: (1) Costanzo et al. (’95b); (2) Packard et al. (’99b); (3) Dinkelacker et al. (2005b); (4) Costanzo et al. (2006); (5) Attaway et al. (’98); (6) Packard and Packard (2004); (7) Willard et al. (2000); (8) Packard and Packard (2003b); (9) Churchill and Storey (’92a); (10) Costanzo et al. (2004); (11) Storey et al. (’88); (12) Packard et al. (2000); (13) Dinkelacker et al. (2004); (14) Baker et al. (2003); (15) Baker et al. (2006); (16) Churchill and Storey (’92b).
tolerance is equivocal. Possibly, this ability enables them to survive brief, shallow freezing episodes that occur in autumn at high latitudes, before nest emergence. Freeze tolerance is weakly developed in hatchling *T. scripta*, which usually overwinter inside the natal nest (Churchill and Storey, '92b; Packard et al., '99b; Dinkelacker et al., 2005b; Costanzo et al., 2006). However, this species is primarily southern in its distribution and has not successfully colonized far northern habitats (Cagle, '50; Holman, '94).

Additional study is needed to clarify phylogenetic, geographic, and ecological associations in chelonian freeze tolerance. The available evidence argues against the hypothesis that the ability to withstand freezing/thawing is widespread among the hatchlings of North American turtles and is a shared ancestral trait (Packard et al., '99b). Rather, it suggests that freeze tolerance has been improved by natural selection for its current role in cold-hardiness strategies in some northern species.

**Evolutionary and ecological implications**

Freeze tolerance and freeze avoidance via supercooling are important mechanisms for coping with subzero temperatures in a diverse array of ectothermic animals (see recent reviews by Ramlov, 2000; Zachariassen and Kristiansen, 2000; Packard and Packard, 2001a; Wharton, 2002). Cryoprotective dehydration is an important strategy of certain small, soil-dwelling invertebrates (Holmstrup, 2003), but has not yet been reported in vertebrates. Although it is widely recognized that freeze tolerance and supercooling modalities are usually mutually exclusive, it remains unclear why a particular taxon adopts one strategy over the other. Some attention has been paid to the costs and benefits associated with these strategies, particularly with respect to energetics, water conservation, and potential for significant cryoinjury (Zachariassen, '85; Block, '91; Duman et al., '95). Voituron et al. (2002a) advanced the theoretical argument that the modality adopted by a particular organism must provide the greatest likelihood of survival whilst also minimizing energy use.

Myriad biotic and abiotic factors influence evolutionary development of cold-hardiness strategies in ectothermic animals. Environmental variation on spatial and temporal scales probably has significant consequences for the genesis of cold-hardiness adaptations (Sinclair et al., 2003b) as, for example, freeze tolerance is a particularly useful strategy in thermally mild and unpredictable environments (Sinclair et al., 2003a). Physical characteristics of the hibernaculum microenvironment, such as factors affecting freezing risk, are particularly important (Ansart and Vernon, 2003), as are certain life-history traits. For instance, body size, which influences capacities for supercooling and freeze tolerance, may strongly influence development of cold-hardiness strategies (Ansart and Vernon, 2003).

Some authors have posited various phylogenetic, ontogenetic, and ecological arguments that freeze avoidance is the basal modality, and that freeze tolerance, which has independently evolved multiple times, is a relatively recent adaptation (Vernon and Vannier, 2002; Sinclair et al., 2003a). There is general agreement that freeze tolerance derives from preexisting, fundamental responses to stresses, such as desiccation and anoxia (Holmstrup, 2003; Costanzo et al., 2006; Storey, 2006), and that its development through natural selection is facilitated in species whose ancestral traits include tolerance to cold and limited freezing (Convey, '97; Sinclair, '99; Voituron et al., 2002a; Sinclair et al., 2004). Research on the winter biology of hatchling turtles has helped elucidate principle mechanisms underpinning the evolution of cold hardiness in ectothermic animals.

**Freeze tolerance or supercooling?**

The painted turtle presents an unusual and interesting example of cold-hardiness evolution in that it can survive chilling episodes through either freeze tolerance or supercooling. This bimodal strategy, which affords plasticity in response to environmental variability, has probably allowed *C. picta* to broadly extend its geographical range. Although sound evidence indicates this animal belongs to the small group of ectotherms whose cold-hardiness repertoire includes both strategies (Stover, '73; Ring, '82; Horwath and Duman, '84; Shimada and Riihimaa, '88; Costanzo et al., '95a; Bale et al., 2001; Voituron et al., 2002a), the literature concerning *C. picta* is marked by competing views of the putative roles of freeze tolerance and supercooling in its winter survival. The laboratory of K. B. Storey, which offered the seminal report of freezing survival in hatchling turtles, advocates freeze tolerance as the major cold-hardiness mechanism in hatchling *C. picta* (Storey et al., '88). Working primarily with northern populations, this research group demonstrated both limited supercooling capacity and profound freeze tolerance, including survival of hatchlings.
exposed to −4°C with over 50% of their body water frozen and of hatchlings kept frozen for at least 11 d (Storey et al., '88; Churchill and Storey, '92a). According to these authors, freeze tolerance in hatchling *C. picta* is a finely tuned adaptation that has evolved from a complex suite of genetic, molecular, and physiological responses to stress (Storey and Storey, '92).

In numerous editorials, G. C. Packard and coauthors (Packard and Packard, '95b, 2001a, 2003c; Packard, 2004a,b) have zealously maintained that supercooling, not freeze tolerance, is the primary survival mechanism of hatchling *C. picta*. These authors initially advocated that hatchlings inside nests are incapable of resisting inoculation and therefore rely on freeze tolerance for winter survival (Packard et al., '89; Packard and Packard, '90). This position was later supplanted by their claim that supercooling, not freeze tolerance, is key to survival, and that turtles must resist inoculation to survive at subzero temperatures. They further asserted that reported instances of freezing survival in hatchling turtles were “nothing more than artifacts of laboratory research” (Packard and Packard, '93b) and that somatic freezing is “fatal to hatchlings, regardless of the minimum temperature to which they are exposed” (Packard and Packard, '95a). More recently, however, these authors acknowledged freezing survival of hatchling turtles, even publishing their own accounts (Attaway et al., '98; Packard et al., '99b; Willard et al., 2000; Packard and Packard, 2003b), but have continued to argue against its adaptive significance. They maintain that the ability to survive somatic freezing is common among chelonians, but is merely an incidental consequence of some other aspect of their biology (Packard et al., '99b; Packard and Packard, 2001a). However, the implication that freeze tolerance arose by happenstance is unlikely considering the unique challenges presented by freezing and thawing stresses and the strong selection pressure needed for evolution of such specific coping mechanisms. Moreover, evidence from several laboratories not only points to definite, species-specific differences in this trait (Costanzo et al., '95b, 2006; Packard et al., '99b; Willard et al., 2000; Dinkelacker et al., 2005b) but also indicates that such variation tends to associate with freezing risk in nature (Dinkelacker et al., 2005b; Costanzo et al., 2006).

Is there any middle ground? Predicated on their 5-yr field and laboratory study, Costanzo et al. ('95b) have promulgated a dual-modality hypothesis. According to this view, supercooling is the predominant strategy when environmental and physiological conditions are conducive to freeze avoidance, but a tolerance for freezing becomes critical in other instances. Subsequent work by these investigators has revealed the complexity of these relationships. Although some authors have considered this hypothesis plausible (Ultsch, '89; Nagle et al., 2000), it has not enjoyed universal acceptance (Packard and Packard, '95a; Weisrock and Janzen, '99).

Difficulty in resolving questions about the putative roles of freeze tolerance and supercooling stems from a paucity of information about the actual physical states of hatchling turtles during natural hibernation. Packard and Packard (2003c) addressed this limitation, stating that it is virtually impossible to obtain such evidence, citing their own failed attempts to examine hatchlings inside frozen nests and to confirm frozen status by distinguishing individual animal exotherms from variation in soil temperatures (Packard, '97). Excavating nests in winter is a challenging undertaking, but can be done, especially if nests are constructed in friable (sandy) soils. Costanzo et al. (2004) extricated *C. picta* from several nests in early February, following an extended period of freezing weather, finding that many were inanimate, ice-covered, and frozen; yet, several revived on warming. Direct determinations of the state of naturally hibernating hatchlings that do not disturb the nest or its occupants would involve methods that have not yet been applied. One possible technique is acoustic sensing, which is based on the premise that sound waves will travel through an unfrozen turtle at a different rate than through a frozen one. Also, indirect methods not previously used to address this question might be brought to bear. For example, the emerging field of metabonomics, a process of characterizing multiparametric responses associated with metabolic dysfunction, may be helpful. Preliminary experiments suggest that the NMR-based metabonomic approach can be used to distinguish tissues that have been frozen from those that have been supercooled (Fig. 15).

Lacking direct observations on free-living animals, investigators have relied on laboratory simulations and measurements of the hibernaculum microenvironment to explain the remarkable cold hardiness exhibited by hatchling *C. picta*. Proponents of the freeze-tolerance hypothesis advocate that, although hatchlings are prone to freezing, their capacity to withstand this stress

*J. Exp. Zool.*
is sufficient to allow recovery from most natural chilling episodes. Proponents of the freeze-avoidance hypothesis argue that, relative to their meager freeze tolerance, frigorific conditions inside hibernacula are too severe and turtles therefore survive winter by virtue of their well-developed capacities to supercool and resist inoculative freezing. Both views draw support from notions about the thermal relations of overwintering hatchlings and their capacities for supercooling, inoculation resistance, and freeze tolerance. However, as we discuss in the following sections, some of this information may be inaccurate or interpreted improperly.

One of the major shortcomings is that direct measurements of body temperature in hibernating turtles are lacking, probably owing to the technical difficulties of collecting such data. In practice, investigators infer body temperature from the output of data loggers placed inside or near the hibernaculum. Although some workers deem these data an acceptable proxy (e.g., Breitenbach et al., '84; Packard, '97), it is noteworthy that hibernacula are three-dimensional structures with complex heating/cooling dynamics and, therefore, actual body temperatures may be higher or lower than those recorded by such devices. In addition, the data output does not necessarily reflect temperatures of all individuals using the same hibernaculum. Strong thermal gradients can occur inside turtle nests (Wilhoft et al., '83; Thompson, '88; Georges, '92; Tucker, '99) and probably cause siblings to experience different thermal conditions (Packard et al., '97a). Additionally, the heat of fusion liberated during freezing of some turtles could, in principle, alter the thermal profiles of nearby individuals. Similarly, cold-hardy slugs are known to "huddle" during chilling, possibly as a means to improve survival (Cook, 2004), and various plants derive protection from the heat of fusion when certain of their parts freeze (Zachariassen and Kristiansen, 2000). Note that the typical 7-g hatchling, having a water concentration of 80% (fresh mass) and an assumed bound water fraction of 20%, liberates /C24 1.0 kJ whilst two-thirds of its body water freezes. Taking values for the specific heat of liquid water (4.18 J g⁻¹ C⁻¹), ice (2.09 J g⁻¹ C⁻¹), and dry hatchling tissue (1.56 J g⁻¹ C⁻¹; Claussen and Zani, '91), we calculate that only 19–25 J, or ≤2.5% of the energy liberated by a single freezing turtle, must be absorbed by an adjacent turtle, either supercooled or frozen, to raise its body temperature 1°C under steady-state thermal conditions. Of course, the nest hibernaculum is incompletely closed to heat exchange and tends to be cooling whilst turtles are freezing. However, considering the insulative properties of frozen soil and the close physical association of the entombed siblings, this scenario seems worthy of study. When considering the cold-hardiness mechanisms of hatchling turtles, workers should recognize the limitations of their methodology and make allowance for imprecision in

Fig. 15. Using a metabonomics approach to distinguish supercooled and frozen states in hatching painted turtles (Chrysemys picta bellii). Results of preliminary experiments, showing (A) representative metabolite profile of brain tissue of a cold-acclimated hatchling, produced by ¹H-NMR spectroscopy in a Bruker 500 MHz NMR spectrometer with an hr-MAS probe and (B) principle components analysis (PCA) suggesting differences in metabolite levels among cold-acclimated (control), supercooled, and frozen/thawed hatchlings. Eggs were collected from gravid females at a nesting area in Garden Co., NE, and incubated in the laboratory at ~29°C. After hatching in August, turtles were kept in darkness and progressively acclimated to 4°C. In December, the control turtle was sampled directly from its cage; other turtles were sampled without rewarming after being held supercooled or frozen for 48 h at −3°C. (G. Lorigan, T. Muir, and P. Baker, unpublished data.)

(with respect to both thermal and temporal limits) is sufficient to allow recovery from most natural chilling episodes. Proponents of the freeze-avoidance hypothesis argue that, relative to their meager freeze tolerance, frigorific conditions inside hibernacula are too severe and turtles therefore survive winter by virtue of their well-developed capacities to supercool and resist inoculative freezing. Both views draw support from notions about the thermal relations of overwintering hatchlings and their capacities for supercooling, inoculation resistance, and freeze tolerance. However, as we discuss in the following sections, some of this information may be inaccurate or interpreted improperly.

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knowing the true thermal relations of hibernating animals.

Claims about the importance of freeze tolerance in the winter life histories of hatchling turtles have been criticized largely on grounds that freeze tolerance is so limited that there could be few instances in which turtles would survive freezing. For example, some authors contend that temperatures in C. picta nests commonly fall below \(-4^\circ C\), the apparent lower limit of freeze tolerance, and that subzero temperatures persist long beyond the temporal limit to survival in the frozen state (Packard and Packard, 2003c). These concerns are not unfounded, but the argument appears overstated for two reasons. First, in assessing the disparity between environmental demands and physiological tolerances, Packard (’97) focused on minimum temperatures and maximum durations of subzero chilling episodes, placing special emphasis on the coldest periods of the coldest winters rather than more routine temperatures and typical durations. Secondly, the principal context for this argument is a C. picta population in northcentral Nebraska, where severe winters can occur owing to a continental climate that is not only very cold but also lacking in insulating snow cover, and yet populations thrive. A more balanced interpretation of the available data is that, even in severe winters, most subzero chilling episodes are relatively benign and, in moderate winters, subzero temperatures occur infrequently, if at all, in many nests (Packard et al., ’89; Paukstis et al., ’89; Costanzo et al., ’95b, 2004; Packard, ’97). It is noteworthy that extreme cold rarely occurs in C. picta nests in the Midwest, Northeast, and other parts of the species’ range, in part owing to the moderating effect of snow cover (Table 2). For example, in multi-year studies of C. picta overwintering in Michigan, Breitenbach et al. (’84) reported that nest temperatures rarely fell below \(-2.0^\circ C\) (although one nest reached \(-3.3^\circ C\)), and Nagle et al. (2000) reported that nests remained frost-free. In one winter in Illinois, only half of the monitored C. picta nests cooled below \(-4^\circ C\) (Weisrock and Janzen, ’99); mortality was slight and occurred only in nests where temperatures fell below \(-8^\circ C\). In northern Indiana, minimum temperatures inside C. picta nests during two consecutive winters, including a severe one, ranged from \(-1.2\) to \(-4.7^\circ C\) (Costanzo et al., 2004). Hatchlings in some populations probably can survive most, if not all, chilling episodes wholly by virtue of their freeze tolerance, limited as it is. Our point here is not to minimize the significance of freezing as a winter mortality factor; rather, we suggest that authors not discount the biological significance of freeze tolerance solely on the basis of generalized claims about the thermal environment inside terrestrial hibernacula.

Capacities for freeze tolerance, supercooling, and inoculation resistance in hatchling turtles are known only from laboratory studies; those of free-living animals have not as yet been determined. Although many of these studies are instructive, results of experiments that were improperly designed or executed have confounded the literature. In particular, failing to control ice nucleation, freezing specimens too rapidly, testing unacclimatized animals, and denying hatchlings adequate recovery time have led authors to underestimate the capacity for freeze tolerance. Furthermore, recent study has shown that thermal history can strongly influence freeze tolerance in some species (Lee et al., 2006b). Supercooling capacity has been overestimated (sometimes grossly) through the practice of hatching and rearing experimental subjects on an artificial, INA-free medium. In tests of inoculation resistance, chilling hatchlings in mock nests without initiating freezing of the soil matrix, as well as constructing nests of exotic clayey soil (rather than indigenous sand), has biased results toward freeze avoidance (Packard and Packard, ’93a,b, ’95a; Packard and Janzen, ’96; Packard et al., ’97c). On the other hand, the convention of completely enveloping individuals in a soil matrix, rather than testing groups of hatchlings in open-chambered nests containing eggshell fragments, probably accentuates freezing risk. Investigators should realize that even the most carefully conducted experiments are crude simulations of the natural environment and that cold-hardiness characteristics of free-living animals may differ considerably from those determined for hatchlings reared and/or tested under laboratory conditions.

**Resolving the debate**

Proponents of the freeze-avoidance hypothesis argue that freeze tolerance is an ineffectual strategy because hibernaculum temperatures can fall below \(-4^\circ C\), the approximate lower lethal temperature for frozen turtles. However, by the same token, supercooling capacity is also limited; that is, hibernaculum temperatures can fall below \(-7\) to \(-8^\circ C\), the critical minimum temperature for turtles hatched in natural nesting soil and cooled...
in perfect isolation from environmental ice and INA (Packard et al., 2001; Costanzo et al., 2003; Packard and Packard, 2003a, 2006). In one field study (Weisrock and Janzen, ’99), winter mortality of hatchling *C. picta* occurred only in nests that fell below −8°C, this threshold matching the lower limit of supercooling for that population (Packard and Janzen, ’96). Otherwise, there is poor concurrence between winter mortality rates and results of laboratory studies delimiting cold hardiness in hatchling turtles (e.g., Packard, ’97; Costanzo et al., 2003, 2004). Neither adaptation, insofar as our current understanding allows, can explain how hatchling *C. picta* successfully emerge in spring from nests that earlier had cooled below −10°C (Woolverton, ’63; DeParri, ’96; Packard, ’97; Packard et al., ’97a; Costanzo et al., 2004), or even −15°C (Costanzo et al., 2003). Clearly, more ecological and physiological information is needed to clarify the roles of these cold-hardiness responses in winter survival. Caution must be used not only in designing suitable experiments, but also in extrapolating laboratory findings to field conditions. The salient relationships are highly complex and investigators will need to exercise patience in elucidating them.

**CONCLUSIONS**

Temperate species of turtles hatch from eggs in late summer. Although the hatchlings of some species leave their natal nest to hibernate elsewhere on land or under water, others remain inside the nest until spring. Most species have an affinity for one behavior or the other, but several exhibit plasticity in this trait, sometimes exhibiting multiple behaviors at the same locale. It is not known why this occurs. Studies addressing physiological regulation of nest emergence behavior in hatchling turtles are lacking. Investigating the endocrine status of emerging and nonemerging hatchlings, and of populations exhibiting both fall and spring emergence might prove rewarding.

Post-hatching behavior strongly influences the winter ecology and physiology of hatchling turtles. The costs and benefits associated with overwintering inside vs. outside the nest have long been contemplated, but the implications of hibernaculum choice remain incompletely understood because fundamental information about hatchling behavior and microenvironmental conditions are lacking. Improvements in radiotelemetry and other tracking techniques would enable investigators to monitor hatchling behavior for sufficiently long periods, providing a more thorough understanding of their winter habits, habitat requirements, and survival rates.

Field biologists need to conduct additional studies of natural hibernacula and environmental conditions confronting hatchling turtles, particularly those overwintering under water. Results of laboratory studies indicate that hatchlings, unlike the older individuals of some species, are unable to survive long-term submergence in anoxic water. Submergence in cold, normoxic water may cause ionic-osmoregulatory perturbations in some species, particularly ones whose hatchlings usually hibernate on land.

Overwintering in terrestrial habitats precludes winter feeding, but hatchlings seem not to be at risk of starvation, probably because their metabolism is markedly reduced during hibernation. Winter mortality is caused by flooding, dehydration, and exposure to severe cold; anoxia is a potential threat, but has not been investigated. Terrestrial hibernators exhibit adaptations that promote survival in these harsh conditions. For example, these species better resist EWL than species whose hatchlings usually hibernate under water; this difference apparently reflects variation in morphology and characteristics of the integument.

Considerable study has been devoted to the cold-hardiness adaptations exhibited by terrestrial hibernators. Hatchlings of all temperate species tolerate a degree of chilling, but survival of repeated, transient exposure to subzero temperatures depends on either freeze avoidance through supercooling or on freeze tolerance, each mechanism having its own advantages and disadvantages. Freeze tolerance permits revival after the tissues have frozen and thawed, but can protect turtles only at modest temperatures (≥−4°C). Supercooling permits survival at much lower temperatures (to perhaps −12°C), but the supercooled state is inherently precarious because turtles are susceptible to inoculation by endogenous and exogenous ice-nucleating agents (INA) and contact with ice in the environment. Freeze tolerance and supercooling usually are dichotomous strategies, but at least one species apparently can switch between them, the one utilized in a particular instance of chilling depending on prevailing physiological and environmental conditions.

Freeze avoidance through supercooling is promoted by behavioral, anatomical, and physiological features that minimize risk of inoculation by ice and INA. Neonates harbor ice-nucleating substances of endogenous origin, but some of
these agents are shed when gut contents are purged. The intrinsic supercooling limit is never reached by hatchlings in nature because freezing is initiated by contact with exogenous agents. Ice is a particularly potent catalyst, but inoculation can also result from contact with various inorganic and organic INA ubiquitously found in terrestrial hibernacula. Susceptibility to inoculative freezing is governed by temperature and various factors that mediate intimacy with these agents, and also by hatching behavior, morphology, and physiology. Generally, terrestrial hibernators exhibit better-developed capacities for supercooling and inoculation resistance than species whose hatchlings usually hibernate under water. In principle, smaller hatchlings are better able to avoid freezing through supercooling and inoculation resistance, and the evidence suggests that hatchlings from populations in cold climates are relatively small. The possibility that the winter environment is a selective force shaping the life-history traits of turtles merits further consideration.

Freeze tolerance is promoted by a complex suite of molecular, biochemical, and physiological responses enabling hatchlings of certain species to survive the freezing and thawing of extracellular fluids. The adaptive strategies of freeze tolerance include control of the ice-nucleation event, management of the redistribution of water and solutes, hypometabolism, upregulation of antioxidant defense systems, and expression of genes involved in homeostasis and somatic repair. Such protective mechanisms probably originated from fundamental responses to osmotic and hypoxic stress.

ACKNOWLEDGMENTS

Many colleagues have provided information and insights concerning the various topics contained herein. We thank them all for their considerable input to this project. We thank G. Lorigan for sharing unpublished data with us, and M. Elnitsky for reviewing an early draft of this article. The submitted paper was strengthened by the commentaries and suggestions offered by J. Iverson and an anonymous referee. The writing, literature review, and some of the research highlighted herein were supported by National Science Foundation Grants (IBN 9817087 and IAB 0416750 to J. P. C., and IBN 00765592 to G. R. U.).

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