# Inoculative freezing promotes winter survival in hatchling diamondback terrapin, *Malaclemys terrapin*

**P.J. Baker, J.P. Costanzo, R. Herlands, R.C. Wood, and R.E. Lee, Jr.**

**Abstract:** We investigated the hibernation ecology and cold hardiness of hatchling diamondback terrapins, *Malaclemys terrapin* (Schoepf, 1793), an estuarine species that reaches 42°N along the Atlantic Ocean. During 3 years of study, about 50% of the nests we monitored harboured hatchlings during winter, and the majority (87%) of these individuals survived despite being intermittently exposed to subfreezing temperatures. Most such exposures were brief (ca. 12 h) and mild (minimum temperature: ca. –1.2 °C); however, turtles were occasionally subjected to longer chilling episodes and lower temperatures. In laboratory experiments, hatchlings supercooled extensively, attaining ca. –15 °C before spontaneously freezing. However, they were highly susceptible to inoculative freezing through contact with external ice and (or) ice-nucleating agents, which occur in nesting soil. Therefore, freeze avoidance through supercooling does not appear to be a viable cold-hardiness strategy in these turtles. Hatchlings subjected to experimental freezing survived exposure to temperatures as low as –3.0 °C, suggesting that freeze tolerance may account for the high winter survival observed in natural nests. We conclude that freeze tolerance in hatchling *M. terrapin* is promoted by high susceptibility to inoculation, which is known to moderate freezing, allowing cells time to adapt to the attendant physical and osmotic stresses.

**Résumé :** Nous avons étudié l'écologie de l'hibernation et de la résistance au froid chez les nouveau-nés de la tortue à dos diamantin, *Malaclemys terrapin* (Schoepf, 1793), une espèce estuarienne dont la répartition atteint 42°N sur les côtes de l'Atlantique. Durant les 3 années de notre étude, environ 50 % des nids que nous avons suivis contenaient des tortues néonates durant l'hiver, dont la plupart (87 %) ont survécu malgré des expositions intermittentes à des températures sous le gel. La plupart des expositions étaient brèves (ca. 12 h) et légères (température minimale: ca. –1,2 °C); néanmoins, les tortues étaient, à l'occasion, exposées à des épisodes de refroidissement plus longs et à des températures plus basses. Dans des expériences de laboratoire, les tortues néonates entrent profondément en surfusion, atteignant ca. –15°C avant de geler spontanément. Elles sont, cependant, très vulnérables au gel par inoculation au contact de la glace externe et (ou) des agents de nucléation de la glace qui sont présents dans le sol du nid. Ainsi, l'évitement du gel par la surfusion ne semble pas être une stratégie viable de résistance au froid chez ces tortues. Des tortues néonates survivent à une exposition à un gel expérimental jusqu'à –3,0 °C, ce qui laisse croire que la tolérance au gel explique probablement la forte survie en hiver observée dans les nids naturels. Nous concluons que la tolérance au gel chez les *M. terrapin* néonates est favorisée par une forte vulnérabilité à l'inoculation, qui, on le sait, provoque un gel lent qui donne aux cellules le temps de s'adapter aux stress physiques et osmotiques concomitants.

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## Introduction

Northern aquatic turtles typically hibernate in the unfrozen depths of ponds and rivers, although the neonates of some species commonly overwinter inside their natal nests. Hibernation within the nest may benefit hatchlings by reducing their exposure to predators at a time when resources necessary for rapid growth in aquatic habitats are in decline

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(Wilbur 1975). However, in northern regions, this strategy demands that the young turtles tolerate exposure to subzero temperatures. Laboratory studies suggest that hatchlings could survive such exposures by avoiding freezing through supercooling (Packard and Packard 1993) or by tolerating the freezing of their tissues (Storey et al. 1988). Although these survival mechanisms appear mutually exclusive, some reptiles apparently can use either strategy, depending on the prevailing physiological and environmental conditions (Costanzo et al. 1995).

Freeze avoidance via supercooling requires that an organism persist in a metastable state in which body fluids are liquid at temperatures below their equilibrium freezing/melting point. In laboratory experiments, the hatchlings of several turtle species exhibited a marked ability to supercool, remaining unfrozen until reaching temperatures between –8 and –20 °C (Costanzo et al. 2000, 2001*b*). Supercooling in these animals is promoted by their small body size and by

their habit of purging from the gut any ingested and (or) endogenously produced matter that might seed the freezing of body fluids (Costanzo et al. 2001*a*, 2003). On the other hand, freezing can be triggered at relatively high temperatures if the supercooled hatchling physically contacts ice or ice-nucleating agents (INA) in the nest microenvironment (Costanzo et al. 2000), so a survival strategy based on supercooling requires effective barriers to the penetration of ice and INA into the body (Packard and Packard 2003).

Regardless of any such anatomical barriers, the likelihood of inoculative freezing in a given hatchling depends on its body temperature and on various attributes of the surrounding soil (Costanzo et al. 1998). For example, soil moisture has a particularly strong influence on inoculation risk because it determines the abundance of ice crystals in the vicinity of the turtle (Baker et al. 2003; Costanzo et al. 1998, 2001*a*, 2004). Soil texture is another important variable and, other factors being equal, hatchlings hibernating in porous, coarse soils, such as sands, are especially susceptible to inoculative freezing (Costanzo et al. 2001*a*). Nesting soils are rife with INA that can trigger freezing in supercooled turtles (Baker et al. 2003; Costanzo et al. 1998, 2000, 2001*a*, 2003, 2004). These agents represent two major classes of INA: inorganic particulates (e.g., quartz and other silicates) and water-soluble, heat-labile agents (e.g., organic detritus and ice-nucleating microorganisms). Environmental moisture regime, soil texture, and the abundance and potency of INA are variables that influence freezing risk in hatchlings that overwinter within their natal nest (Costanzo et al. 2004).

Some organisms lack an ability to supercool extensively, but can tolerate freezing of their body fluids. These "freeze-tolerant" species survive the physical and osmotic stresses associated with the formation of ice within the extracellular spaces of their tissues (Storey and Storey 2004). Few vertebrates are so adapted, although freeze tolerance occurs in some amphibians and reptiles, including several species within the Emydidae, a family of mostly North American pond turtles (reviewed in Baker et al. 2004). These animals can survive only if freezing is initiated at a relatively high temperature and body temperature falls no lower than –3 to –4 °C (Costanzo et al. 1995; Packard et al. 1999; Storey et al. 1988). If these conditions are met, some species such as the painted turtle, *Chrysemys picta* (Schneider, 1783), can recover after >50% of the body water has frozen (Storey et al. 1988) and can tolerate freezing bouts lasting at least 11 d (Churchill and Storey 1992*a*).

The diamondback terrapin (*Malaclemys terrapin* (Schoepf, 1793); family Emydidae) inhabits brackish estuaries and marshes along the Atlantic and Gulf coasts of the United States, from Corpus Christi, Texas (22°N), to Wellfleet, Massachusetts (42°N) (Iverson 1992). Although *M. terrapin* may remain active year round at lower latitudes, in northern regions they become dormant for several months during the winter (Yearicks et al. 1981). Adults hibernate communally in depressions in the mud at the bottom of tidal creeks, in undercut banks within the intertidal zone, or burrow 15–50 cm into the sides of creek banks (Yearicks et al. 1981) and probably escape the extreme cold. Hatchlings do not hibernate among older conspecifics but apparently overwinter within the natal nest (Auger and Giavannone 1979; Roosenburg 1994), where they could encounter frost. To in-

vestigate the functional basis for cold hardiness in hatchling *M. terrapin*, we examined the relationships between the environmental conditions prevailing within their winter refugia and their capacities for supercooling, inoculation resistance, and freeze tolerance.

## Materials and methods

### Study area and characterization of the winter environment

We investigated *M. terrapin* inhabiting a tidal salt marsh in Cape May County, New Jersey (39°N, 74°W), during the three successive winters between 2001 and 2004. The marsh is characterized by salt meadows, narrow creeks, large open sounds, and a broad, relatively deep channel (see Yearicks et al. 1981). Winters in this region are generally wet (>25 cm rain) and cold, although the penetration of frost into the soil may be limited by snow cover and the thermal moderation of the ocean.

We located nests by monitoring the activities of gravid *M. terrapin* along a nature trail at the Wetlands Institute, Stone Harbor, New Jersey, from mid-May to mid-July. We secured a raised, plastic screen above each nest to deter predators and, beginning in August, we visited the nests daily to determine the dates on which the hatchlings emerged. Hatchlings found at the surface were removed from the enclosure, weighed on a digital balance (0.01 g), measured with callipers (0.1 mm; carapace length), and released into the marsh. Site visits were made less frequently in winter, but daily inspections resumed the following spring. In March, we excavated all nests from which at least one hatchling had emerged prior to winter, recording the number and viability status of any eggs and turtles, but deferred examining the remaining nests until the end of the emergence period. In early spring, winter-killed hatchlings are turgid, intact, and have little odour, and thereby can be distinguished from those dying before winter with reasonable certainty (Costanzo et al. 2004).

To characterize the winter thermal environment, we placed miniature temperature loggers (TidBit®; Onset Computer Corp., Pocasset, Massachusetts) within the soil column adjacent to newly constructed *M. terrapin* nests in 2002 (*n* = 2) and 2003 (*n* = 8). These nests were selected in an attempt to represent the diversity of nesting sites at the study area. We assumed that the temperatures recorded by these devices (every 4 h) were similar to those occurring inside the actual nests (e.g., Breitenbach et al. 1984; Vitt and Sartorius 1999). After recovering the data loggers in spring, we analysed the data to determine winter minimum temperature, the number of cooling bouts in which the temperature fell below the equilibrium freezing/melting point of the body fluids (–0.6 °C), and the shortest and longest periods that the temperature continuously remained below this benchmark. Thermal and temporal attributes of these "critical chilling episodes" are useful indices of the severity of cold stress to which the hatchlings were exposed (e.g., Costanzo et al. 1995, 2004; Packard 1997; Packard et al. 1997; Baker et al. 2003).

We tested samples of soil collected ca. 10 cm below ground, adjacent to hatchlings within nests, on 3 March 2003 (*n* = 8) and 19 March 2004 (*n* = 14) to assess the ice-nucleating environment to which the turtles are exposed dur-

ing winter. Soil moisture content was determined from the decrease in mass of a sample during drying in a 65 °C oven and expressed as mass of water per unit mass of dry soil. For the samples collected in 2003, we also determined the relative ice-nucleating activity in two types of INA, following procedures detailed in Costanzo et al. (1998). Briefly, washings containing water-soluble (organic) INA were prepared from the soil, apportioned into 10 µL aliquots ($n$ = 4–6), and chilled at 1.5 °C/min in a refrigerated bath (model RTE 140; Neslab, Portsmouth, New Hampshire) until they spontaneously froze. The temperature of crystallization ($T_c$), averaged for the replicates, was taken as an index of organic INA activity. Similar tests were conducted on soil samples that had been autoclaved to deactivate constituent organic INA. A small volume (ca. 100 mm$^3$) of the treated soil was thoroughly mixed with 12.5 µL of ultrapurified water and chilled until it spontaneously froze. The $T_c$, averaged for $n$ = 6 replicates per soil sample, provided a relative measure of the activity of inorganic, particulate INA in nesting soil.

### Cold acclimation of hatchlings

Gravid *M. terrapin* were captured by hand and induced to oviposit by administering synthetic oxytocin (Ewert and Legler 1978). Eggs collected in this manner were placed on moist vermiculite (1.0 g water/g vermiculite; ca. –150 kPa) in clean, plastic containers. We segregated the eggs by clutch and incubated them at 28 °C in an environmental chamber (model I-35X; Percival, Boone, Iowa). The vermiculite was kept at the initial hydration level by periodically weighing the containers and replacing any water lost by evaporation.

After hatching, in mid-September, turtles were held in darkness inside plastic boxes containing damp vermiculite. They were gradually acclimated to cold using a protocol intended to simulate seasonal changes in temperature in *M. terrapin* nests at the study area. Hatchlings were exposed to 22 °C until 1 October, when the temperature was lowered to 15 °C. Further decrements in ambient temperature were instituted on 1 November (10 °C) and on 1 December (5 °C), and the hatchlings were thereafter held at 5 °C until used in the experiments. Generally, the hatchlings used in each experiment were randomly selected from different clutches to avoid the potentially confounding influence of genetic dependence. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Miami University in accordance with guidelines established by the United States Public Health Service and the Canadian Council on Animal Care.

### Supercooling trials

We investigated innate supercooling capacity in laboratory-reared hatchlings by chilling them and determining the temperature at which they spontaneously froze (Costanzo et al. 1998). On the day before testing, hatchlings were removed from their holding boxes, gently brushed to remove any adhering vermiculite, and held overnight at 5 °C in a dry, loosely covered plastic cup. This procedure permitted evaporation of any surface moisture that might seed the freezing of tissues. We then placed each hatchling upright inside a clean plastic tube (33 mm × 105 mm) and filled the space above it with insulative plastic foam. The tubes were immersed in a refrigerated ethanol bath and, after all turtles

had reached equilibrium at 0 °C, the bath was programmed to cool them at 0.5 °C/h. A thermocouple placed in contact with the turtle's plastron was used to record temperature every 30 s on a data logger (model 3D752; Omega, Stamford, Connecticut) and the $T_c$ was taken as the lower limit of supercooling.

### Inoculation-resistance trials

We examined the ability of the hatchling *M. terrapin* to resist inoculative freezing when chilled in the presence of ice and INA that naturally occur in the nest. The medium used in these trials was a composite of the eight samples of nesting soil (average moisture content: 0.11 g water/g dry soil) collected on 3 March 2003. Turtles were prepared for testing as per the supercooling trials. On the following day, they were placed in plastic tubes to which we added a quantity of soil sufficient to completely immerse the animal, and the tube was closed with a piece of plastic foam. A copper–constantan thermocouple, positioned adjacent to each hatchling, was used to record temperature at 30 s intervals on a data logger (model OM 500; Omega, Stamford, Connecticut). Turtles were habituated within the tubes (5 °C, in darkness) for 24 h. We then submerged the tubes in a refrigerated bath and allowed their contents to equilibrate at –0.4 °C, a temperature at which the soil, but not the turtles, could freeze. Next, we inoculated the supercooled soil with several small ice crystals and permitted it to thoroughly freeze over the next 3–4 h. Finally, the tubes were cooled at 0.5 °C/h until an exotherm (indicating that the turtle inside had begun to freeze) appeared in the temperature recording. The $T_c$ of each turtle was taken as an index of its capacity to resist inoculative freezing under the conditions of the trial.

### Freeze-tolerance trials

We evaluated the capacity of hatchling *M. terrapin* to tolerate somatic freezing by assessing survival of individuals held frozen at various equilibrium temperatures (Costanzo et al. 1995). Hatchlings were prepared for testing as per the inoculation-resistance trials; however, instead of using native soil we immersed them in damp builder's sand (0.15 g water/g dry sand; water potential = –105 kPa) to ensure that all turtles would begin freezing at a temperature near their equilibrium freezing/melting point (see Costanzo et al. 1998). After the sand enveloping the turtles became thoroughly frozen at –0.4 °C, we further cooled the tubes at 0.1 °C/h to the prescribed temperature, i.e., –2.5, –3.0, or –3.5 °C. During this process, a second freezing exotherm invariably appeared in the temperature recording, signifying that the turtle itself began to freeze at ca. –1 °C. Turtles were held at the target temperature for at least 24 h to ensure that an equilibrium ice content was reached, but in some trials we varied exposure duration to probe the limits of freeze tolerance.

Hatchlings were thawed by gradually raising the bath's temperature to 5 °C. We then transferred the turtles to individual cups lined with damp paper and held them at 5 °C in darkness. Viability was assessed by monitoring their response to tactile stimulation and locomotory function during the ensuing 7 d period. Turtles that were incapable of locomotion or otherwise impaired were euthanized.

## Results

### Hatchling behavior, winter survival, and hibernaculum microenvironment

During the 3 years of this study, we examined a total of 148 *M. terrapin* nests, most of which were constructed along a 400 m long nature trail overlying compacted sand and gravel. Generally, females nested in predominantly fine sands complexed with small amounts of organic matter and bivalve shells in sparsely vegetated areas. Each year, several of the monitored nests were depredated or contained eggs that did not hatch. Observations of hatchling behavior and overwintering success were based on the remaining 102 nests, which held between 1 and 20 viable hatchlings. Generally, these turtles emerged from their nests either before winter (5 August through 3 November) or during the following spring (18 April through 21 May); however, in nearly one-third of the cases, clutch mates emerged during both periods (Table 1). Hatchlings emerging in fall 2003 were nominally heavier (6.2 ± 0.1 g (mean ± SE); $n = 363$) than hatchlings emerging in spring 2004 (6.1 ± 0.1 g; $n = 126$), but the difference was not statistically significant (Student's $t$ test, $t = 1.84$, $P = 0.067$).

On average, about half the sample of monitored nests harboured between 1 and 14 turtles during winter and most of these individuals survived to emerge in the spring (Table 2). We found no winter-killed hatchlings in the 6 nests examined in spring 2002. In the following year, winter mortality was limited to 1 of the 6 nests in the sample. This nest contained 10 hatchlings that apparently had died during winter. Following the relatively cold winter of 2003–2004, we found 1–9 winter-killed hatchlings in 9 of the 43 nests that served as hibernacula, and all 9 of these affected nests were ones from which some turtles had emerged the previous autumn. By contrast, survival was 100% for the 15 nests that had spring-only emergence. Overall, mortality occurred in 18% of the nests in which turtles hibernated, and 13% of the overwintering cohort succumbed to winterkill (Table 2).

Temperature recordings were analysed to characterize the thermal conditions to which overwintering turtles were exposed. In 2002–2003, one of the temperature loggers was deployed adjacent to a nest (No. 02-23) within which all eggs failed to hatch. Because the remaining loggers (one in 2002–2003, eight in 2003–2004) were deployed near nests from which some, if not all, hatchlings emerged in the fall, we lack data for nests that remained intact throughout the winter. Winter temperatures within breached nests might have been lower than those in intact nests (e.g., Nagle et al. 2004); nevertheless, six of them contained hatchlings, some of which survived (Table 3).

Our analyses suggest that hatchlings overwintering in the natal nest experienced multiple critical chilling episodes (CCEs), periods of cold exposure when temperature remained below –0.6 °C, which is the approximate equilibrium freezing/melting point of turtle tissues. The typical CCE was relatively brief (ca. 12 h) and mild (minimum temperature: ca. –1.2 °C), but in the extreme, some lasted >100 h and the temperature fell to –4.7 °C (Table 3). Our sample is small, but the available data fail to show the expected association between winter survival and severity of the thermal environment. For example, the sole hatchling occupying nest No. 03-6 died, even though it apparently experienced the fewest and shortest CCEs, and also the mildest temperatures. In contrast, two hatchlings survived inside nest No. 03-22, despite being frequently exposed for long periods to sub-freezing temperatures (Fig. 1).

Samples of soil collected adjacent to eight occupied turtle nests in March 2003 varied in water content but generally were moist (0.12 ± 0.02 g water/g dry soil (mean ± SE); range: 0.08–0.21 g water/g dry soil). Soil samples collected near 14 occupied turtle nests in March 2004 also were relatively moist (0.15 ± 0.01 g water/g dry soil; range: 0.08–0.21 g water/g dry soil). Because soil samples were collected only in March, we have no information concerning moisture levels earlier in winter.

Testing the soil samples for ice-nucleating activity indicated the presence of two classes of potent INA in the winter environment. Washings containing water soluble, presumably organic INA crystallized at –5.7 ± 0.2 °C (mean ± SE; $n = 8$). In contrast, 10 µL samples of ultrapurified (INA-free) water froze at –20.3 ± 0.5 °C ($n = 6$). Soil samples, autoclaved to deactivate constituent organic INA, also exhibited strong ice-nucleating activity (–7.0 ± 0.2 °C; $n = 8$) that probably was associated with inorganic particulates in the soil. An equal volume (12.5 µL) of ultrapurified water (control) crystallized at –19.3 ± 1.5 °C ($n = 6$).

### Supercooling and inoculation resistance

Hatchlings used in tests of supercooling capacity, in which individuals were chilled in a clean, dry environment, froze at –15.1 ± 1.1 °C (mean ± SE; $n = 8$). With the exception of one individual, which froze at –7.0 °C, these turtles avoided spontaneously freezing until they had chilled to temperatures between –12.3 and –18.2 °C. In contrast, hatchlings cooled in a matrix of frozen nesting soil invariably froze ($T_c = -1.1 \pm 0.2$ °C; range: –1.6 to –0.8 °C; $n = 8$) at temperatures just below the equilibrium freezing/melting point of turtle tissues.

### Freezing survival

Turtles readily tolerated exposure to –2.5 °C for at least 7 d; however, none of the turtles held frozen at –2.5 °C for 12 d survived (Fig. 2). Most hatchlings survived freezing at –3.0 °C for 3 d, but none recovered after being held at –3.5 °C for a similar period (Fig. 2). Generally, surviving hatchlings were responsive to tactile stimulation within 12 h of thawing and were capable of locomotion by the 3rd day after thawing. Turtles that succumbed to freezing/thawing showed no response to tactile stimulation or otherwise exhibited neurobehavioral impairment.

## Discussion

Our field study revealed that many hatchling *M. terrapin* routinely and successfully overwinter within their natal nests, even in a population in the northern portion of the species' geographic range. Some hatchlings vacated their nests before winter; however, these individuals probably also hibernate terrestrially, occupying shallow burrows above the high tide line (Draud et al. 2004). In some cases, one or more hatchlings emerged in the fall, whereas the balance of the clutch overwintered inside the nest, possibly with an in-

**Table 1.** Timing of emergence from the nest of hatchling diamondback terrapins (*Malaclemys terrapin*) in Cape May County, New Jersey, during 3 years of study.

|  | 2001–20002 | 2002–2003 | 2003–2004 | Totals (%) |
|---|---|---|---|---|
| All clutch mates emerged before winter |  |  |  |  |
| No. of nests | 10 | 13 | 24 | 24 (46.1) |
| All clutch mates emerged after winter |  |  |  |  |
| No. of nests | 5 | 5 | 15 | 15 (24.5) |
| Clutch mates emerged before and after winter |  |  |  |  |
| No. of nests | 1 | 1 | 28 | 28 (29.4) |
| Totals | 16 | 19 | 67 | 102 (100) |

**Table 2.** Viability status of hatchling *M. terrapin* overwintering in natal nests in Cape May County, New Jersey, during 3 years of study.

|  | 2001–2002 | 2002–2003 | 2003–2004 | Totals |
|---|---|---|---|---|
| Total no. of nests | 6 | 6 | 43 | 55 |
| No. of nests with mortality | 0 | 1 | 9 | 10 |
| No. of nests without mortality | 6 | 5 | 34 | 45 |
|  |  |  |  |  |
| No. of hatchlings overwintering | 57 | 51 | 168 | 276 |
| No. that died | 0 | 10 | 25 | 35 |
| No. that lived | 57 | 41 | 143 | 241 |

**Table 3.** Characteristics of critical chilling episodes (CCE) and thermal conditions associated with overwintering in the nest by hatchling *M. terrapin* in Cape May County, New Jersey, during two winters.

| Nest ID | No. of CCE* | Duration of shortest CCE (h) | Duration of longest CCE (h) | Cumulative CCE duration (h) | Minimum temperature (°C) | Disposition of hatchlings |
|---|---|---|---|---|---|---|
| 2002–2003 |  |  |  |  |  |  |
| 02-23 | 6 | 4 | 136 | 216 | –2.2 | All eggs failed to hatch |
| 02-27 | 1 | 212 | 212 | 212 | –1.6 | All hatchlings emerged in the fall |
| 2003–2004 |  |  |  |  |  |  |
| 03–5 | 10 | 8 | 16 | 128 | –2.3 | Sole overwintering hatchling died |
| 03-6 | 3 | 4 | 8 | 20 | –1.0 | Sole overwintering hatchling died |
| 03-8 | 10 | 4 | 20 | 108 | –2.2 | Sole overwintering hatchling lived |
| 03-10 | 16 | 4 | 20 | 184 | –2.2 | All 10 overwintering hatchlings lived |
| 03-11 | 11 | 4 | 100 | 412 | –4.2 | All hatchlings emerged in the fall |
| 03-12 | 12 | 4 | 108 | 268 | –2.8 | Sole overwintering hatchling died |
| 03-18 | 16 | 4 | 108 | 380 | –4.7 | All hatchlings emerged in the fall |
| 03-22 | 8 | 4 | 132 | 452 | –3.1 | Two of 4 overwintering hatchlings lived |

*Discrete periods of cold exposure when temperature remained below –0.6 °C, the approximate equilibrium freezing/melting point of turtle tissues. A minimum duration of 4 h was assigned to any CCE with a single temperature record below –0.6 °C.

creased risk of mortality. For example, mortality in winter 2003–2004 was limited to nine nests with partial fall emergence. Similarly, Nagle et al. (2004) observed that hatchling turtles hibernating in a nest chamber breached by earlier-emerging siblings suffered higher mortality than hatchlings overwintering in intact nests. Although it remains unknown why some individuals emerge in the fall and others in the spring, it seems clear that this species is well adapted to survive the rigors of terrestrial hibernation. A primary objective of this study was to elucidate the mechanistic basis for cold hardiness in these turtles.

## Supercooling and inoculative freezing

Hatchling *M. terrapin* exhibited a marked ability to supercool, as most in the sample remained unfrozen until chilled to ca. –15 °C. The supercooling capacity exhibited by hatchling *M. terrapin* was comparable with that of hatchlings of other terrestrially hibernating species reared and tested identically (Costanzo et al. 2001*b*; Baker et al. 2003; Dinkelacker et al. 2004). Therefore, turtle hatchlings apparently possess neither ice-nucleating proteins nor endogenous INA that instigates freezing at relatively high temperatures (Costanzo et al. 1998, 2003). Although these results could indicate that supercooling is important to winter survival, caution must be used in extrapolating laboratory findings to natural situations. Studies have shown that the supercooling capacity of hatchling turtles raised on (INA-free) vermiculite, as were the *M. terrapin* in this study, is much greater than that of individuals hatched and (or) reared on actual nesting soils (Costanzo et al. 1998, 2003). In nature, turtles

**Fig. 1.** Temperatures associated with two diamondback terrapin (*Malaclemys terrapin*) nests during January and February 2004. Hatchlings overwintering inside these nests were at risk of freezing whenever the temperature fell below the equilibrium freezing/melting point of turtle tissues (broken line). When examined in March 2004, nest No. 03-10 contained 10 live hatchlings, and nest No. 03-22 held two live hatchlings and two winter-killed hatchlings.
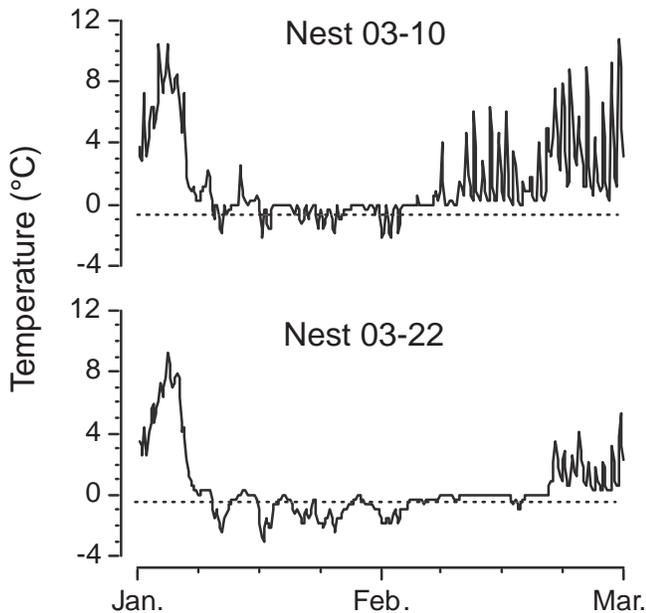


invariably become contaminated with environmental INA during hatching and, consequently, supercooling capacity can be reduced by 50% or more (Costanzo et al. 2000, 2003; Packard and Packard 2003). Testing the supercooling capacity of hatchlings raised on artificial media can reveal the presence or absence of endogenously produced INA; however, evaluating the possible role of supercooling in cold hardiness requires the use of ecologically relevant tests of inoculative resistance.

Inoculative freezing occurs when ice or INA external to an organism causes its tissues to freeze. Mechanisms of transmission of these agents to the tissues of hatchling turtles are incompletely understood. The integument is thought to be an important barrier in hatchling turtles (Packard and Packard 1993; Packard et al. 1993; Willard et al. 2000; Costanzo et al. 2001*b*), although ice and (or) INA nevertheless could access the body fluids via the nares, eyes, mouth, cloaca, and umbilicus (Costanzo et al. 2001*a*, 2003).

Hatchling *M. terrapin* cooled in a matrix of frozen nesting soil began to freeze at temperatures very near the equilibrium freezing/melting point of their body fluids, suggesting that they are highly susceptible to inoculative freezing. The environment in which hatchling *M. terrapin* overwinter promotes inoculative freezing for several reasons. First, our results indicate that nesting soil harbours an abundance of ice crystals and potent INA. Samples of soil collected near turtle nests were relatively moist, suggesting that the turtles are in proximity to ice, which is an extremely efficient catalyst of tissue freezing (Willard et al. 2000). These samples also contained at least two forms of INA that expressed ice-nucleating activity within typical ranges and probably be-

long to the same classes of organic and inorganic INA occurring in turtle nesting soils (and other terrestrial habitats) in diverse geographic regions (Costanzo et al. 2000, 2001*a*). Relative to ice, these agents are probably of secondary importance in causing supercooled turtles to freeze; however, their relative importance increases when environmental moisture is limited (Costanzo et al. 2000).

Another factor contributing to inoculation susceptibility in hatchling *M. terrapin* is their habit of overwintering in coarse-textured soils, such as sands, that often contain little organic matter and few colloids. Although sandy soils drain more readily than clays and loams, their high porosity tends to increase exposure to ice crystals and INA (Costanzo et al. 2001*a*, 2004). Freezing risk is particularly high for hatchlings that overwinter in sandy soils that are frequently moist (Costanzo et al. 2001*a*), although apparently some riverine species (e.g., *Graptemys geographica* (Lesueur, 1817)) have overcome this challenge (Baker et al. 2003; Nagle et al. 2004).

Inoculation resistance in hatchling turtles varies taxonomically, perhaps reflecting differences in body size and morphology, which determine the expanse of exposed skin (Costanzo et al. 2001*b*; Baker et al. 2003; Dinkelacker et al. 2004), and (or) the ultrastructural characteristics of the skin itself (Willard et al. 2000). We wished to compare inoculative resistance in *M. terrapin* to that in other species. However, because inoculative freezing is so strongly influenced by environmental conditions, comparisons among studies are valid only if experimental protocols are identical. Therefore, we tested additional hatchling *M. terrapin* using a standardized medium (9:1 mixture of loamy sand and fine clay, hydrated to 0.075 g water/g dry mass; water potential: ca. –400 kPa) that was used in earlier studies of other taxa (Costanzo et al. 1998, 2001*b*; Baker et al. 2003; Dinkelacker et al. 2004). In these experiments, hatchling *M. terrapin* began to freeze at –1.7 ± 0.5 °C (*n* = 8). This value is ~10 °C higher than values determined for *C. picta* and *G. geographica*, two species that can employ supercooling to survive chilling episodes (Costanzo et al. 2001*a*; Baker et al. 2003), but is similar to values obtained for the hatchlings of several other species, including freeze tolerant ones (Costanzo et al. 2001*a*; Dinkelacker et al. 2004). We cannot exclude the possibility that hatchling *M. terrapin* remain supercooled during brief, relatively mild chilling episodes, but our findings strongly suggest that their ability to resist inoculative freezing under usual environmental conditions is extremely limited.

**Freeze tolerance**

*Malaclemys terrapin* can be considered a freeze-tolerant species (sensu Baust 1991) given that our hatchlings survived experimental freezing under conditions of ecological relevance. These turtles can recover from freezing at –2.5 °C for at least 7 d (but not as long as 12 d) and can tolerate temperatures between –3.0 and –3.5 °C. Given the thermal environment prevailing inside nests during hibernation (Table 2, Fig. 1), as well as the high winter survivorship (Table 1), this level of tolerance seems adequate to protect hatchlings during most chilling episodes. It remains to be determined whether populations from higher latitudes, which

**Fig. 2.** Results of freeze-tolerance trials in which hatchling *M. terrapin* were exposed to –2.5, –3.0, or –3.5 °C for periods lasting 2–12 d. Each bar depicts the number of live and dead hatchlings in each experiment.
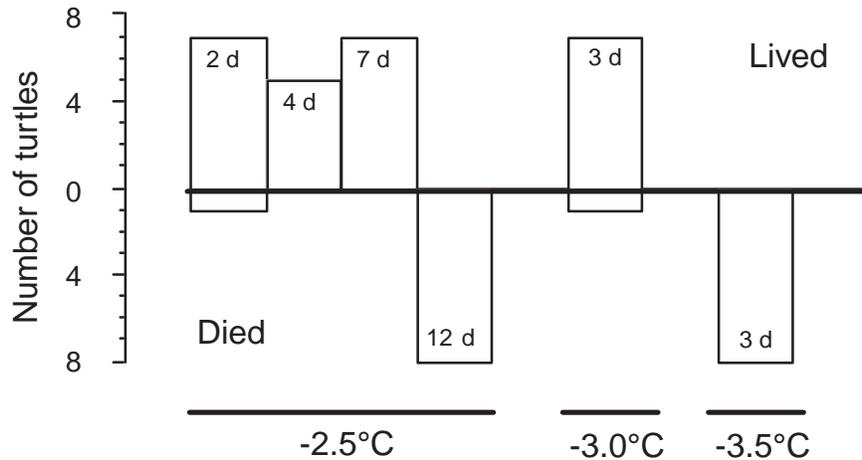


presumably experience lower winter temperatures, have an enhanced cold hardiness or suffer higher mortality rates.

The capacity for freeze tolerance in hatchling *M. terrapin* apparently exceeds that exhibited by some emydids but is lower than that found in others. For example, our *M. terrapin* tolerated freezing at lower temperatures and for longer periods than did hatchling *G. geographica* (Baker et al. 2004) and *Trachemys scripta* (Schoepff, 1792) (Churchill and Storey 1992*b*; Packard et al. 1999). On the other hand, whereas hatchling *C. picta* can survive freezing at –2.5 °C for at least 11 d (Churchill and Storey 1992*a*), in this study *M. terrapin* did not tolerate freezing at this temperature for 12 d. Furthermore, unlike the case with our *M. terrapin*, hatchling *Emydoidea blandingii* (Holbrook, 1838) readily recover from exposure to –3.5 °C for 3 d (Dinkelacker et al. 2004). Further research is needed to ascertain the limits of freeze tolerance in hatchling *M. terrapin*, and this work should consider that freeze tolerance in hatchling turtles varies seasonally and may be fully expressed only if animals are field acclimatized (see Costanzo et al. 2004).

Little is known about the physiological mechanisms underpinning reptilian freeze tolerance, so it is difficult to determine what factors set the thermal and temporal limits to freezing survival in hatchling turtles. In principle, lactate accumulation and energy depletion could impact freezing endurance because, as ice propagates throughout the body, cardiac function ultimately ceases and the ischemic, frozen tissues must rely on anaerobic glycolysis to meet metabolic demands (Rubinsky et al. 1994). Some workers (Packard and Packard 2004) have conjectured that the accumulation of lactate, the main end product of glycolysis, constrains freezing survival, but recent work (Dinkelacker et al. 2005) has shown that hatchling turtles tolerate lactate loads much higher than that achieved during freezing. Alternatively, death may result when the supply of glucose, primarily produced in the liver, no longer supports the cellular demands for ATP (Layne et al. 1998).

Our hatchling *M. terrapin* tolerated somatic freezing at equilibrium body temperatures as low as –3.0 °C, but exposure to –3.5 °C invariably was lethal, possibly reflecting the formation of an exceedingly large quantity of ice within the tissues. Generally, freeze-tolerant animals tolerate only the

freezing of up to two-thirds of their body water (Storey and Storey 2004). Although ice contents of our turtles were not determined, based on calorimetric data reported for other emydids (Churchill and Storey 1992*a*, 1992*b*), we estimated that ~40%–55% of the body water had frozen in hatchling *M. terrapin* exposed to –2.5 °C. Cooling these turtles to −3.5 °C could have increased their ice contents beyond the survival threshold.

## Ecological and evolutionary perspectives

Freezing survival is promoted by mechanisms that control the distribution of ice and retard its propagation in the tissues. Generally, ice nucleation must occur at a temperature near the tissue equilibrium freezing/melting point in order that cells can adapt to attendant physical and osmotic stresses (Lee and Costanzo 1998; Storey and Storey 2004). Some freeze-tolerant organisms synthesize ice-nucleating proteins or sequester environmental INA that trigger freezing, but hatchling turtles apparently do not (Costanzo et al. 1998, 2001*b*, 2003). The relatively poor resistance to inoculative freezing found in some freeze-tolerant vertebrates, including *M. terrapin*, may fulfill this role. Because hatchling *M. terrapin* are morphologically similar to emydids that are highly resistant to inoculative freezing, variation in this trait probably reflects differences in the ultrastructural characteristics of the integument or other barriers to the infiltration of environmental ice or INA.

Freeze tolerance in certain reptiles might also be promoted by mechanisms regulating cell volume during dehydration and osmotic challenge (Storey and Storey 2004). If this was the case, then freeze tolerance as a cold-hardiness adaptation might have evolved from fundamental responses to challenges presented by desiccating environments (Churchill and Storey 1993; Costanzo et al. 1993; Lee et al. 1992). *Malaclemys terrapin* is an interesting taxon in this regard because its tolerance for hyperosmotic aquatic habitats is unparalleled among emydid turtles (Dunson 1970). These turtles become profoundly dehydrated during exposure to seawater, yet they tolerate this condition for extended periods (Bentley et al. 1967; Gilles-Baillien 1970). Using this species in future studies of the link between freeze tolerance and adaptations to osmotic stress may prove rewarding.

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