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## COLD HARDINESS AND OVERWINTERING STRATEGIES OF HATCHLINGS IN AN ASSEMBLAGE OF NORTHERN TURTLES<sup>1</sup>

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**Abstract.** Field and laboratory studies were conducted during 1989–1994 to investigate the overwintering strategies of hatchling turtles representing four families native to western Nebraska. Whereas hatchling snapping turtles (*Chelydra serpentina*) and spiny soft-shelled turtles (*Apalone spinifera*) overwinter in aquatic habitats, yellow mud turtles (*Kinosternon flavescens*) and ornate box turtles (*Terrapene ornata*) burrow below the natal nest and hibernate in sandy soil. Painted turtles (*Chrysemys picta*) overwinter within their shallow natal nests, but this species, and *T. ornata*, tolerate extensive tissue freezing. Overwintering behaviors of these species are consistent with indices of physiological cold hardiness and patterns of geographic distribution. Frost commonly penetrated and persisted below 10 cm, the soil depth at which hatchling *C. picta* routinely hibernate. Field and laboratory data suggested that hatchling *C. picta* survive either by remaining supercooled (unfrozen) or by tolerating tissue freezing, the strategy employed depending on prevailing physiological and microenvironmental conditions. Whereas relatively lower temperatures can be survived in the supercooled state, supercooling capacity may be limited via the inoculation of body fluids by environmental ice. Alternatively, whereas freeze tolerance fortuitously is promoted by ice inoculation, this strategy may be viable only at relatively high subzero temperatures. A cold-hardiness strategy based on both survival mechanisms may promote winter survival in hatchling *C. picta* by conferring protection under dynamic physiological and microenvironmental conditions. Physiological cold hardiness and behavior are integrated determinants of the northern distributions of temperate region turtles.

**Key words:** adaptation; biogeography; freeze tolerance; hatchling turtle; ice inoculation; micro-environment; Nebraska sandhills; overwintering behavior; supercooling; winter survival.

### INTRODUCTION

Turtles are long-lived animals whose tolerance of heat, cold, desiccation, and hypoxia permits them to inhabit stressful environments. Because natural mortality rates are greatest for hatchlings (Wilbur 1975, Iverson 1991a), this age class has been well studied by both physiologists and ecologists. In cold temperate regions, hatchlings of some species are particularly vulnerable to overwintering mortality, a result that significantly limits recruitment and constrains population size (e.g., MacCulloch and Secoy 1983, St. Clair and Gregory 1990). Investigation of the overwintering strategies of northern turtles may elucidate not only the demographic ramifications of life in extreme environments, but also the ecophysiological factors influencing species' distributions.

Overwintering of hatchlings in the nest has been confirmed for five turtle families indigenous to all continents having cool temperate climates (Gibbons and

Nelson 1978). One benefit of overwintering and emerging from the nest in spring, rather than autumn, is that hatchlings enter an environment of increasing resources, such as heat, light, and food (Wilbur 1975, Ultsch 1989). However, for most species this behavior becomes increasingly uncommon at higher latitudes, perhaps because the hazards of overwintering on land outweigh the benefits of spring emergence. Thus, in northern populations hatchlings may abandon the nest in autumn and, like the adults, hibernate in relatively protected aquatic habitats.

One notable exception to this pattern is the painted turtle (*Chrysemys picta*), whose hatchlings routinely overwinter in the natal nest, even at the northern limit of its range (Ultsch 1989). Low temperature is likely the most critical factor influencing their winter survival (e.g., Breitenbach et al. 1984, St. Clair and Gregory 1990), although desiccation also may be important (Christiansen and Gallaway 1984, Packard et al. 1989). Winter temperatures inside *C. picta* nests commonly fall below zero and may reach lows of  $-5^{\circ}$  to  $-11^{\circ}\text{C}$

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(Woolverton 1963, DePari 1988, Storey et al. 1988, Packard et al. 1989).

Survival of subzero winter temperatures by hatchling *C. picta* initially was attributed to supercooling (Bailey 1949, Bleakney 1963, Breitenbach et al. 1984), a physical property allowing organisms to remain unfrozen when body temperature ( $T_b$ ) declines below the tissues' equilibrium freezing point ( $FP_{eq}$ ). This supposition is espoused by some current workers (DePari 1988, Paukstis et al. 1989, Packard and Packard 1993), although others attribute overwintering success to freeze tolerance, a remarkable adaptation that permits recovery from tissue freezing (Storey et al. 1988, Churchill and Storey 1992a, b). Given that the lowest  $T_b$  survived by frozen hatchlings under laboratory conditions is  $\approx -4^\circ\text{C}$  (e.g., Churchill and Storey 1992a), and that supercooling may be constrained by inoculation through contact with ice in the environment (e.g., Costanzo and Lee 1995), the precise roles of freeze tolerance and supercooling under natural conditions are unknown. A fuller comprehension of this species' cold hardiness strategy requires a more thorough investigation of the constraints of freeze tolerance and supercooling in the context of the microenvironmental conditions it encounters during hibernation.

We investigated patterns of cold hardiness in an assemblage of five turtle species, representing four families, native to western Nebraska. Hatchlings of these species collectively exhibit three distinct overwintering strategies: aquatic hibernation, terrestrial hibernation deep under ground, and terrestrial hibernation within the shallow natal nest. Our primary objective was to investigate overwintering strategies relative to nest emergence behavior, physiological cold hardiness, and microenvironmental conditions. Ontogenetic and geographic comparisons were provided through laboratory studies of the cold hardiness of adult conspecifics and hatchlings from a more temperate, midwest locale. Additional studies were purposed to evaluate supercooling and freeze tolerance as potential cold hardiness mechanisms in hatchling *C. picta*, which owing to its habit of overwintering within the nest, likely exhibits a higher degree of cold adaptation than any other species in North America.

## MATERIALS AND METHODS

### *Study area and field measurements*

The study area is near Gimlet Lake ( $41^\circ\text{N}$ ,  $102^\circ\text{W}$ ) on the Crescent Lake National Wildlife Refuge (NWR), Garden County, Nebraska (Iverson 1990, 1991b). The predominant habitat is rolling, mid-grass prairie punctuated by shallow lakes and ephemeral ponds lying in swales among sandhills and occasional clear, spring-fed streams. Climate in this region is characterized by cold, dry winters; warm, wet springs; hot, dry summers; and cool, dry autumns. Approximately half of the 44 cm annual rainfall occurs May–July. Native che-

lonians are the painted turtle (*Chrysemys picta*), common snapping turtle (*Chelydra serpentina*), spiny soft-shelled turtle (*Apalone spinifera*; found only in streams), yellow mud turtle (*Kinosternon flavescens*), and ornate box turtle (*Terrapene ornata*).

Soil temperatures were recorded at the study area during a 20-wk period (20 October 1990–3 March 1991) and a 26-wk period (15 October 1993–10 April 1994) at a representative location on the south-southwest face of a sandhill traditionally used by nesting *C. picta* and *K. flavescens*, and probably *T. ornata*. Thermistors buried in the soil at depths of 5–25 cm (5-cm intervals) were used in conjunction with a digital data recorder (model 1206-42 Squirrel; Science/Electronics, Dayton, Ohio, USA) to log temperature every 2 h. The probes were routed through a protective conduit to an underground junction box from which they emerged, traversed 70 cm laterally at the prescribed depth, and were anchored via attachment to an upright wooden staff. During the course of this study, turtles continued to nest (and overwinter) at this site, occasionally within 0.5 m of the probes. Breitenbach et al. (1984) found no difference between winter temperatures inside *C. picta* nests and those of the soil adjacent to nests at corresponding depths; presumably our soil temperatures also reflect thermal conditions encountered by hibernating turtles.

For each soil depth we enumerated and characterized potential freezing episodes (PFE), operationally defined as a discrete sequence of soil temperatures  $\leq -0.55^\circ\text{C}$ , the approximate  $FP_{eq}$  of turtle tissues (e.g., Churchill and Storey 1992a, b). Individual PFEs were measured for duration, minimum temperature, and maximum cooling rate, the latter calculated from successive temperature recordings. Additionally, we totaled the number of PFE hours for the entire sample period and distributed these data among various temperature classes. Air temperature, precipitation, and snow cover were recorded by Crescent Lake NWR personnel at headquarters,  $\approx 1$  km distant. Climatic data for the periods of study were compared to average values representing the last 15 winters.

The geometry and depth of nests containing *C. picta* eggs were measured during June and July 1990 ( $n = 6$ ) and July 1992 ( $n = 2$ ). Soil collected from the cavities of other *C. picta* nests in 1990 ( $n = 4$ ) and 1993 ( $n = 12$ ) during mid-October was analyzed for water content by oven drying; data were expressed both on a mass basis (milligrams water per gram dry soil) and as a percentage of saturation, determined from the mass of deionized water held against gravity. Water potential of soil from turtle nests at field water content was measured with a stainless steel psychrometer (Wescor, PST-55, Logan, Utah, USA) and dew-point microvoltmeter (Wescor, HR-33T, Logan, Utah, USA) coupled to a MacLab data acquisition system. These measurements, determined for a pool of soil equally representing all 12 nests sampled in 1993, were made using standard

protocols and appropriate NaCl standards. Samples from this pool were also used in analyses of the physical properties of soil within the nest. Particle size analyses of six 1993 samples, selected at random, were conducted by the Soil and Plant Analysis Laboratory, University of Wisconsin-Madison, Madison, Wisconsin.

Observations of the overwintering behavior of hatchling turtles were made on autumn and early spring visits to the study area during each year of the study (1989–1994). In 1990 and 1993, cylinders of wire mesh fabric (15 cm diameter  $\times$  61 cm length) were implanted vertically within the soil column such that they contained and protected turtle clutches from predators (chiefly moles, *Scalopus aquaticus*, hognose snakes, *Heterodon nasicus*, and various carnivores). Cylinders protecting nests of *K. flavescens* and *T. ornata* (both of which do not emerge from the sandhills until spring) were capped by a wire screen positioned  $\approx$ 2.5 cm beneath the soil surface, but were open at the lower end. Cylinders encircling nests of *C. picta* and *C. serpentina* were open on both ends. The location of hatchlings relative to the nest cavity was determined during autumn and/or spring, and the timing of emergence from the dunes was discerned from the appearance of hatchlings abroad.

#### *Specimens used in laboratory studies*

In 1989 and 1990, hatchlings were obtained either from laboratory-incubated eggs (*C. picta*, *T. ornata*, *A. spinifera*, and *C. serpentina*) or marked natal nests (*C. picta* and *K. flavescens*) excavated immediately before (October) or after (April–May) hibernation. Eggs, harvested from females administered oxytocin, were handled and incubated following the protocol of Etchberger et al. (1992). Experiments requiring sexed hatchlings (*C. picta*) used specimens emerging from eggs incubated at 25°C (probable males) or 30°–32°C (probable females). For comparison, we studied *C. picta* hatchlings produced by a single female from Richmond County, Indiana collected from a nest during November 1989. Some tests were conducted using adult turtles (*K. flavescens* and *T. ornata*) captured in early spring 1990 shortly after emerging from hibernation.

All specimens were maintained unfed in plastic cages housed in an environmental chamber. Unless otherwise specified, water was provided to deter desiccation. Turtles hatching in the laboratory (in early September) initially were kept at 20°C, but subsequently conditioned to low temperature before being tested. This regimen involved lowering the chamber temperature from 20° to 15°C in mid-October and subsequently from 15° to 4°C in early November. Additional hatchlings (and adults) were field collected during cool weather in early spring or autumn and so were directly exposed to 4°C. All specimens were kept at 4°C in constant darkness for 8–16 wk before testing.

#### *Cooling protocol and terminology*

Hatchlings were blotted dry, instrumented with a 36-gauge, copper-constantan thermocouple whose measuring junction was glued to the carapace center, and insulated with a small overlying patch of plastic foam. Specimens were placed individually inside dry, 50-mL plastic tubes, which were wrapped in several layers of plastic foam and submerged in a refrigerated bath. Following the protocol of Costanzo and Claussen (1990), adult turtles were fitted with a thermocouple probe positioned  $\approx$ 2 cm inside the cloaca and cooled in an insulated jar submerged in a cold bath. A multichannel recorder (Omega Electronics, OM-500, Stamford, Connecticut, USA) logged  $T_b$  during cooling. From these data we determined the time supercooled, commencing when the specimen reached the equilibrium tissue  $FP_{eq}$ ,  $-0.55^\circ\text{C}$ , and ending at either the onset of ice crystallization or the trial's conclusion. If tissue freezing occurred, the onset of ice crystallization was marked by an exothermic response to the change in physical state of body water. The  $T_b$  at which ice crystallization began ( $T_c$ ) represents the lowest  $T_b$  attained during supercooling. Freeze duration is the interval commencing at ice crystallization and ending with the removal of the specimen from the bath at the trial's conclusion. Minimum  $T_b$  is the ultimate, lowest  $T_b$  attained during supercooling or freeze tolerance trials.

#### *Freeze tolerance*

Turtles were cooled by gradually lowering the bath temperature from 0°C to the prescribed target temperature. Ice crystallization of hatchlings was induced ( $T_c \approx -1^\circ\text{C}$ ) by introducing small ice crystals inside the tube in the proximity of the specimen. It was unnecessary to stimulate freezing in the (larger) adult turtles, as they commonly begin freezing with little or no supercooling (Costanzo and Claussen 1990, Costanzo and Lee 1995). Nevertheless, the initiation of freezing without prior supercooling was heralded by the distinct cessation of cooling and stabilization of  $T_b$  near the tissue  $FP_{eq}$ . Specimens were kept frozen at least 24 h during which time they cooled and ultimately attained the target minimum  $T_b$  (usually  $-2.5^\circ\text{C}$ ). Subsequently, they were passively thawed at 4°C in a darkened environmental chamber. We judged that turtles had fully recovered if normal behaviors (limb and head retraction reflexes, body posture, and spontaneous locomotion) were restored during a 2-wk postthaw observation period.

#### *Supercooling capacity of *C. picta**

Supercooling capacity was determined for hatchling *C. picta* (mean  $\pm$  1 SE =  $3.6 \pm 0.1$  g;  $n = 18$ ) randomly assigned to groups and cooled individually inside dry, 50-mL plastic tubes to a target minimum  $T_b$  of  $-2^\circ$ ,  $-4^\circ$ , or  $-8^\circ\text{C}$ . Cooling rate (0.5°C/h) was closely regulated by a temperature programmer (Neslab Instru-

ments, ETP 3, Newington, New Hampshire, USA), since variation in this parameter may confound interpretation of these results (Costanzo and Lee 1995). Turtles attaining the target minimum  $T_b$  without freezing remained in situ at the prescribed temperature for several days. However, trials in which specimens froze were concluded shortly after ice crystallization began.

#### *Cold hardiness of desiccated C. picta*

We reassessed supercooling capacity in one treatment group ( $-8^{\circ}\text{C}$  trial) after hatchling *C. picta* were partially desiccated by housing them over  $\text{CaCl}_2$  ( $4^{\circ}\text{C}$ ) for 3 wk. The influence of body water content on cold hardiness mechanisms was further investigated in *C. picta* by using additional hatchlings in trials of supercooling (mean  $\pm 1$  SE =  $3.2 \pm 0.2$  g;  $n = 9$ ) and freeze tolerance ( $2.8 \pm 0.1$  g;  $n = 8$ ). These specimens, collected from natal nests in October 1991, were permitted to dehydrate freely by maintaining them at  $4^{\circ}\text{C}$  in substrate originating from their nest chamber, without access to water. After testing, body water content was determined from the mass lost on drying carcasses at  $65^{\circ}\text{C}$ . For comparative purposes, water contents were also determined for fully hydrated specimens.

#### *Susceptibility of C. picta to inoculative freezing*

Hatchling *C. picta* weighing  $3.9 \pm 0.2$  g (mean  $\pm 1$  SE,  $n = 14$ ) were cooled in a homogeneous mixture of soil pooled from four nest chambers, dried, and rehydrated to various moisture levels ranging from 0 to 80% of saturation (260 mg water/g dry soil); water potential of the experimental substrates was determined as previously described. Hatchlings were instrumented with an insulated thermocouple and placed centrally within a 4-cm column of prepared substrate ( $\approx 90$  g) inside a plastic cylinder. An additional thermocouple was positioned in the soil column at the depth of the turtle. Contact between the turtle and this probe, whose function was to detect freezing of the soil, was prevented by an intervening plastic screen.

The cylinder was cooled in a glass jar submerged in a refrigerated bath initially set at  $4^{\circ}\text{C}$  and subsequently cooled to  $-2.5^{\circ}\text{C}$ . To prevent extensive supercooling of the substrate, ice nucleation was induced by applying a 5-mm layer of crushed ice to the soil surface when the soil attained a temperature of  $0^{\circ}\text{C}$ . However, to assess the rapidity of the response, in some trials both substrate (80% of saturation) and turtle were first supercooled to  $-2^{\circ}\text{C}$  before the ice was applied.

Exotherms associated with ice crystallization of the substrate and turtle invariably were detected by the respective probes; however, because the heat liberated by freezing soil might be misconstrued as an animal exotherm, we verified the frozen status of each specimen at the trial's conclusion. Hatchlings, with thermocouple intact, were rapidly freed of adhering soil, inspected for rigid appendages and warmed to room

temperature inside an insulated cup. The presence of rigid limbs (which are pliable in supercooled hatchlings) and an endothermic warming curve (associated with the melting of tissues) was taken as further evidence that the specimen had frozen.

#### *Statistical inferences*

Thermal and temporal aspects of PFEs occurring at different soil depths were compared using Kruskal-Wallis tests; multiple contrasts followed Dunn's procedure. Kendall's rank correlation was used to evaluate associations between certain PFE parameters, and also between clutch size and physical dimensions of turtle nests. Values of  $T_c$  from turtles before and after experimental dehydration were compared using Wilcoxon matched-pairs tests. Mean data are reported  $\pm 1$  SE. The results of statistical procedures (following Zar 1984) were regarded significant at  $P \leq 0.05$ .

## RESULTS

### *Overwintering of turtles at the study area*

Hatchling *C. picta* remained in their natal nests from the time they hatched (presumably early August to late September) until the time they emerged, usually mid-April. Nests ( $n = 8$ ) investigated prior to the hatching of eggs were constructed in soft loamy sand, with the cavity's center positioned  $10.6 \pm 0.5$  cm (range, 7.6–12.2 cm) below ground surface. Clutches ( $15.3 \pm 0.8$  eggs; range, 12–19) occupied a chamber  $5.5 \pm 0.4$  cm (range, 4.0–7.0 cm) high, whose roof was located  $7.8 \pm 0.6$  cm (range, 4.1–9.3 cm) under ground. Clutch size was not linearly associated with nest chamber height (Kendall's  $\tau = 0.15$ ;  $P > 0.50$ ) or depth of the chamber's center below ground ( $\tau = -0.54$ ;  $P = 0.061$ ). However, the roofs of nests containing larger clutches were located closer to the ground surface ( $\tau = -0.63$ ;  $P = 0.030$ ).

On 20 October 1990, 10 of 20 *K. flavescens* hatching within three protected nests were found  $55 \pm 3$  cm (range, 41–66 cm) below the ground surface, directly beneath the nest cavity, indicating that they were descending for overwintering. Lacking evidence of egg failure or nest predation, we presume that unrecovered individuals (which must have dug at least 63.5 cm below ground surface to escape from the protective cylinder) were located even deeper. On 15 October 1993, six *T. ornata*, the viable products of 10 eggs, were also located directly below protected natal nests,  $56 \pm 3$  cm (range, 50–68 cm) under ground. Hatchling *K. flavescens* and *T. ornata* remained underground during winter and ultimately emerged in early May. In contrast, most hatchling *C. serpentina* (and presumably *A. spinifera*; Ultsch 1989) emerged from the sandhills during late September; however, a few individuals, observed within natal nests during mid-October, likely would have overwintered on land.

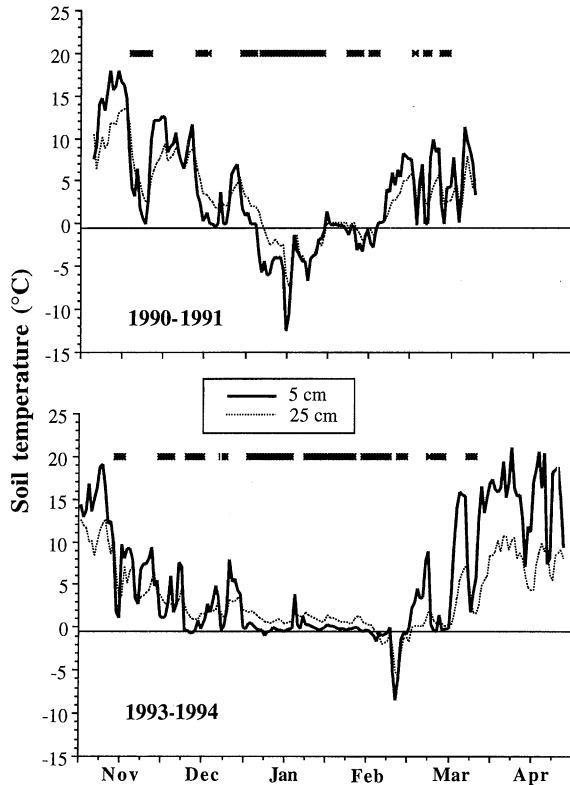


FIG. 1. Soil temperatures recorded at 5 and 25 cm depths within a sandhill at a traditional turtle nesting and overwintering site in Garden County, Nebraska. Temperature was sampled 12 times daily; however, for clarity, only the value sampled each noon was plotted. The approximate  $FP_{eq}$  of turtle tissues,  $-0.55^{\circ}\text{C}$ , is denoted by the continuous horizontal line. Broken horizontal bars indicate the presence of snow cover.

#### Winter microenvironment at the study area

The winter of 1990–1991 was moderately cold. Average daily air temperatures for December ( $-7.6^{\circ}\text{C}$ ) and January ( $-6.2^{\circ}\text{C}$ ) were several degrees below the mean values for these months,  $-3.8^{\circ}$  and  $-4.0^{\circ}\text{C}$ , respectively, for the last 15 yr. However, the lowest air temperatures measured in December ( $-38.3^{\circ}\text{C}$ ) and January ( $-23.9^{\circ}\text{C}$ ) fell within the long-term range of minimum winter temperatures,  $-15.6^{\circ}$  to  $-43.3^{\circ}\text{C}$ . Precipitation as rainfall and snowfall during October–February amounted to 7.2 and 73.7 cm, respectively.

During winter 1990–1991, PFEs occurred most frequently during a 6-wk period extending from mid-December to late January (Fig. 1). The frequency of PFEs decreased markedly with increasing soil depth, as did the duration of individual PFEs (Kruskal-Wallis  $H = 12.2$ ;  $P = 0.02$ ). PFEs lasted from 2 h to  $>13$  d, but generally were  $\approx 24$  h. The cumulative period of freezing risk ranged from 646 h (27 d) at 25 cm to 862 h (36 d) at 5 cm, 19.4 and 25.8%, respectively, of the entire period of record (Table 1).

Soil temperatures vacillated during winter, particu-

larly at shallow depths and during protracted PFEs. The lowest soil temperatures occurred during late December (Fig. 1). The minimum temperatures attained during individual PFEs commonly were  $\geq -3.5^{\circ}\text{C}$ , although extreme cold (e.g., to  $-13.6^{\circ}\text{C}$ ) occasionally occurred, particularly at shallow soil depths (Table 1). Unexpectedly, median values for minimum PFE temperature did not vary with soil depth ( $H = 4.13$ ;  $P = 0.39$ ), however, the range values for this parameter indicate that absolute minimum temperatures were less severe at greater soil depths. Milder temperatures prevailed during PFEs occurring deeper in the soil. For example, whereas  $\approx 40\%$  of PFE temperatures at 5 cm were  $< -4.6^{\circ}\text{C}$ , at 25 cm this frequency was only 10% (Fig. 2).

Cooling and warming cycles in the soil generally followed a diurnal pattern that was most pronounced near the surface. Cooling patterns were analyzed to determine the highest cooling rate associated with each PFE (Table 1). These values, typically  $-0.2$  to  $-0.5^{\circ}\text{C}/\text{h}$ , decreased ( $H = 32.6$ ;  $P = 0.001$ ) and generally became less variable with increasing soil depth. The highest cooling rate,  $-1.6^{\circ}\text{C}/\text{h}$ , occurred 5 cm below the soil surface during a PFE initiated on 3 January 1991.

With respect to air temperature, the winter of 1993–1994 was typical for the study site. Average daily air temperatures for December and January were  $-1.4^{\circ}$  and  $-4.8^{\circ}\text{C}$ ; the minimum record for the winter,  $-25^{\circ}\text{C}$ , was comparable to the long-term average. Snowfall during October–February was 73.7 cm. Rainfall during this period amounted to a record 10.7 cm, 32% higher than average.

PFEs occurred sporadically from late November to early March (Fig. 1) and less frequently at greater soil depths (Table 1). However, soil depth was not statistically associated with PFE duration ( $H = 4.20$ ;  $P = 0.38$ ), minimum temperature ( $H = 2.88$ ;  $P = 0.58$ ), or maximum cooling rate ( $H = 3.84$ ;  $P = 0.43$ ). Despite the relatively mild air temperatures, PFEs were more common in winter 1993–1994 at soil depths of 5 and 10 cm than in 1990–1991 (Table 1). Generally, however, these were of shorter duration and with more moderate minimum temperatures. The temperature of the soil at a depth of 10 cm, where hatchling *C. picta* overwinter, was  $\geq -2.6^{\circ}\text{C}$  for  $\approx 50\%$  of the total period of freezing risk (Fig. 2).

Correlation analyses involving parameters of PFEs occurring at a depth of 10 cm indicated that PFE duration was inversely correlated with minimum temperature in both 1990–1991 ( $\tau = -0.72$ ,  $P < 0.001$ ) and 1993–1994 ( $\tau = -0.75$ ,  $P < 0.001$ ). Additionally, there was a strong inverse association between minimum PFE temperature and maximum cooling rate in 1990–1991 ( $\tau = -0.54$ ,  $P = 0.008$ ) and 1993–1994 ( $\tau = -0.86$ ,  $P < 0.001$ ). Thus, some PFEs were relatively severe in that they were characterized by long duration, rapid cooling, and low temperatures.

TABLE 1. Temporal and thermal attributes of potential freezing episodes (PFE) occurring within a sandhill during cold (1990–1991) and typical (1993–1994) winters at a traditional turtle nesting site in Garden County, Nebraska. A PFE is a discrete sequence of soil temperatures  $\leq -0.55^\circ\text{C}$ , the approximate equilibrium freezing point (FPEq) of turtle tissues.

PFE parameter*	Depth from soil surface (cm)				
	5	10	15	20	25
1990–1991					
Frequency	35	14	12	10	9
Duration (h)					
Mean $\pm$ SE	25 $\pm$ 9	54 $\pm$ 25	58 $\pm$ 28	71 $\pm$ 34	72 $\pm$ 36
Median	12 <sup>a</sup>	23 <sup>a</sup>	25 <sup>a</sup>	32 <sup>b</sup>	29 <sup>a</sup>
Range	2 to 302	6 to 320	6 to 306	10 to 302	6 to 284
Cumulative	862	760	692	712	646
Min. temperature ( $^\circ\text{C}$ )					
Mean $\pm$ SE	-3.9 $\pm$ 0.5	-4.4 $\pm$ 0.8	-3.7 $\pm$ 0.7	-3.2 $\pm$ 0.8	-2.6 $\pm$ 0.8
Median	-2.9 <sup>a</sup>	-3.4 <sup>a</sup>	-3.1 <sup>a</sup>	-2.7 <sup>a</sup>	-2.0 <sup>a</sup>
Range	-13.6 to -0.6	-11.8 to -0.8	-9.8 to -1.1	-8.6 to -1.4	-7.4 to -0.8
Max. cooling rate ( $^\circ\text{C}/\text{h}$ )					
Mean $\pm$ SE	0.58 $\pm$ 0.06	0.52 $\pm$ 0.06	0.35 $\pm$ 0.03	0.26 $\pm$ 0.02	0.18 $\pm$ 0.02
Median	0.45 <sup>a</sup>	0.58 <sup>a</sup>	0.37 <sup>ab</sup>	0.25 <sup>b</sup>	0.18 <sup>b</sup>
Range	0.20 to 1.63	0.90 to 0.13	0.15 to 0.55	0.20 to 0.40	0.08 to 0.30
1993–1994					
Frequency	66	16	7	5	5
Duration (h)					
Mean $\pm$ SE	11 $\pm$ 1	14 $\pm$ 5	21 $\pm$ 12	27 $\pm$ 15	30 $\pm$ 20
Median	8 <sup>a</sup>	8 <sup>a</sup>	10 <sup>a</sup>	14 <sup>a</sup>	12 <sup>a</sup>
Range	2 to 88	2 to 86	2 to 90	6 to 88	4 to 108
Cumulative	720	216	148	134	148
Min. temperature ( $^\circ\text{C}$ )					
Mean $\pm$ SE	-2.3 $\pm$ 0.2	-2.5 $\pm$ 0.6	-3.2 $\pm$ 0.9	-3.3 $\pm$ 1.0	-2.5 $\pm$ 0.9
Median	-1.8 <sup>a</sup>	-1.6 <sup>a</sup>	-2.2 <sup>a</sup>	-2.9 <sup>a</sup>	-2.2 <sup>a</sup>
Range	-11.8 to -0.8	-9.6 to -0.8	-8.2 to -0.7	-7.2 to -1.5	-5.9 to -1.0
Max. cooling rate ( $^\circ\text{C}/\text{h}$ )					
Mean $\pm$ SE	0.40 $\pm$ 0.03	0.36 $\pm$ 0.07	0.45 $\pm$ 0.09	0.42 $\pm$ 0.06	0.26 $\pm$ 0.04
Median	0.34 <sup>a</sup>	0.33 <sup>a</sup>	0.42 <sup>a</sup>	0.45 <sup>a</sup>	0.30 <sup>a</sup>
Range	0.07 to 1.3	0.05 to 0.85	0.10 to 0.72	0.28 to 0.57	0.15 to 0.35

\* Within rows, group medians identified by different lowercase superscript letters were statistically distinguishable ( $P \leq 0.05$ ).

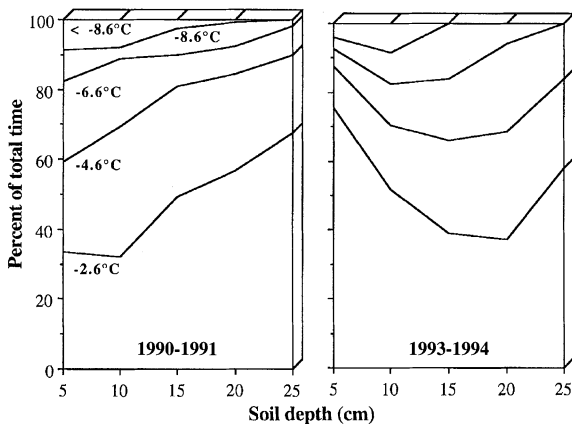


FIG. 2. Temporal characterization of potential freezing episodes (PFEs) occurring within a sandhill at a traditional turtle nesting and overwintering site in Garden County, Nebraska. Illustrated are the distributions of total PFE time at each soil depth among selected temperature classes, which are distinguished by isotherms.

Water contents of soil from *C. picta* nests sampled in mid-October were  $16.6 \pm 1.2$  mg/g ( $7.1 \pm 0.5\%$  of capacity;  $n = 4$ ) in 1990 and  $67.5 \pm 6.1$  mg/g ( $23.6 \pm 2.1\%$  of capacity;  $n = 12$ ) in 1993. These differences reflect variation in the precipitation occurring June–October (20.0 cm in 1990, 43.3 cm in 1993). Estimated water potential at field water content was  $-725$  kPa in 1990 and  $> -50$  kPa in 1993. These soils were light (bulk density =  $1.6$  g/cm<sup>3</sup>; particle density =  $2.6$  g/cm<sup>3</sup>; porosity =  $39.8\%$ ), well-drained sands or loamy sands (sand,  $90.8 \pm 0.5\%$ ; silt,  $1.2 \pm 0.2\%$ ; clay,  $8.0 \pm 0.5\%$ ;  $n = 6$ ).

*Freeze tolerance in hatchling turtles*

Of the five species studied, only *T. ornata* and *C. picta* survived discriminatory tests of freeze tolerance in which specimens attained minimum  $T_{fS}$  of  $-2.5^\circ$  to  $-3^\circ\text{C}$  (Table 2). Some hatchling *T. ornata* did not fully

TABLE 2. Experimental parameters and survival rates of hatchling and adult turtles used in discriminatory tests of freeze tolerance.

Species	No. surviving/no. tested	Body mass (g)		Minimum $T_b$ (°C)*		Freeze duration (h)†	
		$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
<b>Hatchling</b>							
<i>Chrysemys picta</i>	6/6	3.9	0.6	-2.6	0.1	25.5	0.6
<i>Terrapene ornata</i>	2/5	9.9	0.9	-2.6	0.1	36.0	4.2
<i>Kinosternon flavescens</i>	0/5	3.1	0.3	-2.6	0.1	26.3	0.7
<i>Apalone spinifera</i>	0/6	5.9	0.2	-3.0	0.2	33.1	5.1
<i>Chelydra serpentina</i>	0/3	9.1	0.2	-2.6	0.2	49.3	0.0
	0/3	9.6	0.5	-4.0	0.1	48.0	0.0
	3/3	9.1	0.1	-1.5	0.1	28.6	1.4
<b>Adult</b>							
<i>Terrapene ornata</i>	4/4	330	17	-2.8	0.1	73.2	13.4
<i>Kinosternon flavescens</i>	0/4	317	35	-2.6	0.1	96.9	5.6

\* Lowest equilibrium body temperature.

† Interval between the appearance of the exotherm and the trial's conclusion.

meet our recovery criterion, although all adults did. The lower thermal limit of freezing survival for hatchling *C. picta* was investigated by freezing additional specimens, including some native to Indiana, at  $-3^\circ$  to  $-5^\circ\text{C}$ . The apparent lethal threshold,  $\approx -3^\circ\text{C}$ , was independent of (presumed) sex or geographic origin (Table 3). Hatchlings of *A. spinifera* and *K. flavescens*, and adults of *K. flavescens*, were moribund following thawing (Table 2), whereas hatchling *C. serpentina* initially recovered some neuromuscular functions, but died 10 d after thawing. In follow-up studies, three hatchling *C. serpentina* frozen at  $-4.0^\circ\text{C}$  were dead on thawing, whereas three others, having attained a minimum  $T_b$  of  $-1.5^\circ\text{C}$ , rapidly and completely recovered.

#### Supercooling capacity of hatchling *C. picta*

Hatchling *C. picta* reached the target minimum  $T_b$  of  $-2^\circ\text{C}$  without freezing and remained supercooled at this temperature for  $\approx 3$  d before the trials were concluded (Table 4). In contrast, specimens cooled to lower target  $T_b$ s invariably froze (Table 4). Thus, hydrated hatchlings, which contained  $77.2 \pm 0.6\%$  water (mass/

mass;  $n = 8$ ), supercooled only modestly. Six specimens (used in the  $-8^\circ\text{C}$  trial) retested after partial desiccation remained supercooled  $5.6 \pm 0.1$  h and crystallized at  $-3.4^\circ \pm 0.1^\circ\text{C}$ . Accordingly, a reduction in body water ( $74.9 \pm 0.2\%$ ;  $n = 6$ ) of  $\approx 12\%$  resulted in a greater supercooling duration (Wilcoxon  $\Sigma$  rank = 0;  $P < 0.02$ ) and reduced  $T_c$  (Wilcoxon  $\Sigma$  rank = 0;  $P < 0.02$ ).

Hatchling *C. picta* kept over winter in natural substrate, without access to water, showed a marked capacity for supercooling. Of nine turtles used in supercooling trials, freezing occurred in only one (which did not recover); eight specimens attained minimum  $T_b$ s between  $-5.7^\circ$  and  $-8.6^\circ\text{C}$  ( $-6.9 \pm 0.4^\circ\text{C}$ ) and remained supercooled for the trials' duration,  $32.0 \pm 0.2$  h. Except for one individual (minimum  $T_b = -8.1^\circ\text{C}$ ), all supercooled turtles rapidly recovered after warming. Freeze tolerance was investigated in eight other hatchlings from this group, but none of these individuals, frozen  $30.8 \pm 5.0$  h at a minimum  $T_b$  of  $-7.8 \pm 0.2^\circ\text{C}$  (range,  $-7.2^\circ$  to  $-8.6^\circ\text{C}$ ), survived. The mean body water content of turtles in this group,  $69.9 \pm 0.8\%$  ( $n$

TABLE 3. Experimental parameters and survival rates of hatchling painted turtles (*C. picta*) used in discriminatory tests of freeze tolerance.

Target $T_b$ (°C)	No. surviving/no. tested	Minimum $T_b$ (°C)*		Freeze duration (h)†		Origin	Sex‡
		$\bar{X}$	SE	$\bar{X}$	SE		
-3.0	1/2	-2.9	0.1	30.8	0.0	Nebraska	male
-3.5	0/7	-3.5	0.1	30.7	0.8	Nebraska	male
	0/8	-3.6	0.1	37.8	3.0	Nebraska	female
-4.0	0/8	-4.0	0.01	30.8	3.3	Nebraska	mixed
	0/3	-4.2	0.1	24.0	0.0	Indiana	unknown
-5.0	0/5	-4.9	0.03	24.0	0.0	Nebraska	mixed
	0/4	-5.0	0.1	24.3	0.0	Indiana	unknown

\* Lowest equilibrium body temperature.

† Interval between the appearance of the exotherm and the trial's conclusion.

‡ Presumed from the temperature to which eggs were exposed during laboratory incubation; Indiana specimens were incubated under unknown field conditions.



TABLE 4. Results of laboratory tests of supercooling capacity with hatchling painted turtles (*C. picta*) cooled to various target minimum body temperatures. Means are shown  $\pm 1$  SE (range).

	Target minimum $T_b$ ( $^{\circ}\text{C}$ )		
	-2.0	-4.0	-8.0
No. freezing/no. tested	0/6	6/6	6/6
Time supercooled (h)	67*	4.4 $\pm$ 0.2 (3.9 to 4.8)	4.3 $\pm$ 0.1 (3.9 to 4.6)
$T_c$ ( $^{\circ}\text{C}$ )†	...	-3.0 $\pm$ 0.1 (-3.2 to -2.7)	-2.8 $\pm$ 0.1 (-3.0 to -2.6)
Body mass (g)	3.6 $\pm$ 0.2	3.6 $\pm$ 0.1	3.5 $\pm$ 0.1

\* No specimen froze; all trials were concluded after 67 h.

† Lowest temperature attained during cooling before the onset of ice crystallization.

= 17), was 31.4% lower than that of hydrated specimens.

#### *Inoculative freezing of hatchling C. picta*

Hatchling *C. picta* surrounded by dry soil from natal nests readily supercooled, attaining the target minimum  $T_b$  ( $-2.5^{\circ}\text{C}$ ) and remaining unfrozen until the trials were concluded; thus, no exotherm was detected by either the substrate or animal probe. In contrast, specimens tested in freezing substrates containing even small quantities of water invariably themselves froze (Table 5). Trials involving the nucleation of supercooled substrates (at  $-2^{\circ}\text{C}$ ) revealed that crystallization of turtle tissues began virtually concomitantly with the freezing of the substrate, an indication that environmental ice rapidly propagates across the integument. All turtles survived these tests.

#### DISCUSSION

Whereas terrestrial turtles invariably hibernate on land, the overwintering behaviors of freshwater species of North American chelonians are more complicated. The hatchlings of some aquatic species, which hatch in subterranean nests during late summer, may spend their first winter on land, either within or below the natal nest. Such delayed emergence both introduces hatchlings to favorable environmental conditions (Gibbons and Nelson 1978) and reduces predation mortality during winter (Wilbur 1975). However, for most species the propensity for this behavior declines with in-

creasing latitude, perhaps because the increased risk of freezing mortality outweighs the benefits of delayed emergence (Gibbons and Nelson 1978, Ultsch 1989). In northern climates, hatchlings typically abandon the nest in autumn and seek refuge in the presumably more thermally moderate aquatic hibernacula as commonly used by adults (Ultsch 1989).

Of notable exception is *C. picta*, whose hatchlings routinely overwinter in the nest, even in Canada, near the species' northern limit (e.g., Bleakney 1963, MacCulloch and Secoy 1983). In cool climates terrestrial overwintering may be necessitated by retarded embryo development and the consequent, delayed hatching (Sexton 1957, Gibbons and Nelson 1978) or a physical inability to penetrate the encrusted overlying soil in autumn (Hartweg 1944, Bleakney 1963, DePari 1988). Alternatively, terrestrial overwintering may be facultative if, by virtue of its exceptional cold hardiness, northern *C. picta* retains the ecological benefits of spring emergence (Ultsch 1989).

Nest emergence behaviors of northern turtles vary, apparently in accordance with species differences in the ability to avoid frost or tolerate subzero  $T_b$ . At our study area in western Nebraska, hatchlings of *A. spinifera* and *C. serpentina*, which do not survive extensive tissue freezing, commonly emerge from natal nests in autumn and hibernate under water. Hatchling and adult *K. flavescens*, which are freeze intolerant, overwinter terrestrially but hibernate well below the frost line (Iverson 1991b). The freeze-tolerant hatchlings of *C. picta* and *T. ornata* also overwinter in sandhills,

TABLE 5. Ice crystallization temperatures of substrate and hatchling painted turtles (*C. picta*) during cooling to  $-2.5^{\circ}\text{C}$  in the laboratory, relative to substrate water parameters. Means are shown  $\pm 1$  SE.

No. freezing/ no. tested	$T_c$ ( $^{\circ}\text{C}$ )*		Substrate water		
	Substrate	Hatchling	Content (mg/g)†	% of capacity‡	Potential (kPa)
0/4	...	...	0	0	$\infty$
4/4	-0.4 $\pm$ 0.04	-0.6 $\pm$ 0.1	22.8	8.8	-72
4/4	-0.3 $\pm$ 0.1	-0.7 $\pm$ 0.1	68.8	26.6	>-50
2/2	-2.0 $\pm$ 0.0	-2.0 $\pm$ 0.0	208.2	80.2	>-50

\* Lowest temperature attained during cooling before the onset of ice crystallization.

† Mg water per gram dry soil.

‡ Based on the mass of deionized water held against gravity.

TABLE 6. Survival of hatchling painted turtles (*C. picta*) overwintering in natal nests relative to the minimum temperatures to which they were exposed.

Study locale	No. nests observed	Hatchling survival (%) <sup>*</sup>	Minimum nest temperature (°C) <sup>†</sup>	Sample period	Reference
St. Louis County, Minnesota	1	100	-11	Nov-May‡	Woolverton 1963
E. S. George Reserve, Michigan	9	100	-3.3 (9)	Nov-Apr	Breitenbach et al. 1984
Algonquin Park, Ontario	3	100	-6, -8 (2)	Jan-Feb‡	Storey et al. 1988
Great Swamp N.W.R., New Jersey	1	100	-10.5	Sep-Apr	DePari 1988
	1	100	-5.5	May-Dec	DePari 1988
Valentine N.W.R., Nebraska	7	22-100	-0.2 to -6.2 (7)	Nov-Feb‡	Packard et al. 1989
Kikomun Creek Park, British Columbia	3	0-10	-6 (1)	Jun-Mar	St. Clair and Gregory 1990
	8	0	-5 (1)	Jun-Apr	St. Clair and Gregory 1990

<sup>\*</sup> Percentage of fully formed hatchlings within individual nests alive in spring.

<sup>†</sup> Minimum temperatures measured within or adjacent to nest cavities during the sample period for which corresponding survival data are presented. For studies involving more than one nest, the number of nests thermomtered is given in parentheses.

<sup>‡</sup> Discontinuous sampling design probably underestimated true minimum temperature.

although only the former remains in natal nests where extremely low temperatures occur.

#### Soil temperatures during winter

We believe that our soil temperatures reflect the thermal conditions encountered by at least some terrestrially hibernating turtles for two reasons. First, during the course of the study turtles nested and overwintered in high densities in the vicinity, occasionally within 0.5 m of the temperature probes. Secondly, during the winter of 1993-1994 we recorded soil temperatures (depth = 10 cm) adjacent to two turtle nests in the warmest winter microenvironments available to hatchling *C. picta* at the study site. Located along the south wall of a large heated storage building, these nests attained absolute minimum temperatures of  $-7.7^{\circ}\text{C}$  (western end) and  $-5.5^{\circ}\text{C}$  (eastern end), values comparing favorably with the lowest temperature recorded at the exposed sandhill,  $-9.6^{\circ}\text{C}$ .

Despite the presence of snow cover, which substantially moderates nest temperature (Obbard and Brooks 1981, Breitenbach et al. 1984), subzero temperatures penetrated  $\geq 25$  cm, well below the depth at which hatchling *C. picta* overwinter. Potential freezing episodes (PFEs) were less frequent (but typically prolonged) at greater soil depths, where thermal minima and cooling rates were relatively moderate. This buffering effect of the overlying soil is clearly illustrated by the delay and diminished amplitude of cooling and heating profiles of soil at a depth of 25 cm relative to that at 5 cm (Fig. 1).

The nest chambers in which hatchling *C. picta* overwintered were shallow ( $\approx 10$  cm below ground surface) and well within the frost zone. During the cold winter of 1990-1991, hatchlings probably encountered 14 discrete PFEs of which half were fairly mild (i.e., minimum temperatures  $\geq -3.4^{\circ}\text{C}$ ). Substantially lower soil temperatures prevailed during some PFEs, albeit for

short periods. During the more typical winter of 1993-1994, hatchlings probably encountered about the same number of PFEs, but these were relatively mild with respect to both minimum temperature and duration (Table 1). The proportion of total PFE time during which soil temperatures  $\geq -2.6^{\circ}\text{C}$  prevailed was  $\approx 50\%$  in 1993-1994 as compared to  $\approx 30\%$  in 1990-1991 (Fig. 2).

Our record low temperature at 10 cm ( $-11.8^{\circ}\text{C}$ ), the depth where *C. picta* overwinters, was lower than those reported for many other *C. picta* nests, although Woolverton (1963) recorded a winter minimum of  $-11.1^{\circ}\text{C}$  in a nest from which hatchlings emerged the following spring (Table 6). Inter-study variability in minimum nest temperatures may partly owe to differences in methodology (e.g., the point-sampling method undoubtedly underestimates thermal minima), but likely also reflects differences in climate, topographic, or substrate conditions, such as prevailing temperature, soil type, vegetative cover, and snow cover. Judging from the abundance of hatchlings abroad the following spring (and assuming that soil temperatures accurately represent nest temperatures), our 1990-1991 field data suggest that hatchling *C. picta* can indeed survive winter temperatures of  $\approx -12^{\circ}\text{C}$ .

Although our discussion heretofore assumes that hatchlings overwinter in the nest's center,  $\approx 10$  cm beneath the soil surface, in actuality the nest chamber occupies a space 4-7 cm in height. Thus, given the sharp vertical gradient in PFE frequency, thermal minima, and cooling rate at these shallow depths (Table 1; Fig. 2), clutch mates may experience markedly different microenvironmental conditions during winter. This variation conceivably may account for the differential winter survival observed within some nests (e.g., MacCulloch and Secoy 1983, DePari 1988, Packard et al. 1989, Lindeman 1991).

*Freeze tolerance as a cold-hardiness strategy in northern turtles*

Freeze tolerance in reptiles was initially reported for the garter snake, *Thamnophis sirtalis* (Costanzo et al. 1988), and hatchling *C. picta* (Storey et al. 1988). Adult *C. picta* are also freeze tolerant (Claussen and Zani 1991), a potentially beneficial attribute because their aquatic hibernacula do not necessarily preclude exposure to subzero temperatures (e.g., Christiansen and Bickham 1989). Some freeze-tolerant reptiles can survive long-term freezing of >58% of their total body water (Costanzo and Claussen 1990). The physiological basis for reptilian freeze tolerance is poorly understood (Costanzo et al. 1988, 1993, Storey et al. 1988, Churchill and Storey 1992a, b).

Freeze-tolerant vertebrates survive tissue freezing only within a narrow  $T_b$  range. For example, hatchling *C. picta* from Ottawa, Canada, can survive freezing at  $-4^{\circ}\text{C}$ , but not at  $-11^{\circ}\text{C}$  (Storey et al. 1988). Furthermore, whereas this species tolerates 3–11 d of continuous freezing at  $-2.5^{\circ}\text{C}$ , survival time decreases to 4–5 h during freezing at  $-4^{\circ}\text{C}$  (Churchill and Storey 1992a). These findings generally accord with our assessment of the survivable minimum temperature of hatchlings from western Nebraska,  $\approx -3^{\circ}\text{C}$ . Our data for Indiana specimens suggest that there may be little geographic variation in the freeze tolerance capacity of hatchling *C. picta*, although a definitive test of this hypothesis should include specimens from more southern populations.

Contending that the findings of freeze tolerance studies do not reflect the physiology of *C. picta* in nature, but rather represent artifacts of laboratory research, Packard et al. (1993:151) recently stated “. . . the laboratory observations that hatchlings have a limited tolerance to freezing are not especially relevant to the ecology of the animals.” Indeed, temperatures below the survivable minimum of frozen animals occurred in both winters of record in the present study, indicating that supercooling was essential for survival. However, it is not implausible that freeze tolerance capacity permits hatchlings to survive at least some PFEs. For example, during the typical winter of 1993–1994, turtles, had they frozen, may have readily survived 13 of the 16 PFEs that were characterized by relatively moderate minimum temperatures ( $-1.6 \pm 0.2^{\circ}\text{C}$ ) and durations ( $7.4 \pm 1.1$  h) since these parameters were within this species' range of freeze tolerance (i.e., survival at  $T_b$ s of  $-3^{\circ}$  to  $-4^{\circ}\text{C}$  for at least several days). During mild winters in our study area, and at more temperate locales, freeze tolerance may have considerable ecological relevance. In a Michigan study, in which the minimum recorded nest temperature was only  $-3.3^{\circ}\text{C}$  (Breitenbach et al. 1984), winter survival of hatchling *C. picta* could be wholly attributed to either freeze tolerance or supercooling.

Our discovery of freeze tolerance in hatchling and

adult *T. ornata* validates the supposition of Legler (1960), who, noting the superficial hibernacula of some Kansas *T. ornata*, speculated that they survive exposure to subfreezing temperatures. Despite their freeze tolerance, hatchling *T. ornata* did not hibernate in the natal nest, but rather burrowed far beneath it. Similarly, in southern Wisconsin, near the species' northeastern limit, hatchlings avoid low nest temperatures (e.g.,  $-8^{\circ}\text{C}$ ) during winter by descending in the sandy soil during autumn (Doroff and Keith 1990). The adults also hibernate deep under ground (average, 70 cm; range, 50–180 cm) but nevertheless may encounter frost, which on average penetrates to 60–70 cm (Doroff and Keith 1990). Freeze tolerance, coupled with burrowing behavior (and appropriately friable soils), may permit *T. ornata* to survive in such climates. Interestingly, *T. carolina*, an eastern congener, is also freeze tolerant (Costanzo and Claussen 1990, Costanzo et al. 1993), but is restricted to more moderate climates where it hibernates in comparatively shallow burrows.

Like *T. ornata*, the amphibious *K. flavescens* routinely hibernates on land as hatchlings (Christiansen and Gallaway 1984, Long 1986, Iverson 1991b) and adults (Christiansen and Bickham 1989, Tuma 1993). This species, presumably having evolved in xeric environments, is adept at burrowing because it occupies ephemeral aquatic habitats (Iverson 1990, 1991b). Unlike *T. ornata*, however, *K. flavescens* clearly lacks freeze tolerance; thus, both hatchlings and adults must burrow deeply to avoid frost. At our study area, hatchlings evacuate the subterranean natal nest in autumn (probably soon after hatching), descend in the soil, and ultimately overwinter at depths  $\geq 70$  cm. Ten hatchlings, produced in three separate nests, were found  $54.6 \pm 2.7$  cm below soil surface on 20 October 1990; thus, burrowing must occur very soon after hatching. Tuma (1993) reported that adult *K. flavescens* in Illinois hibernated in former aestivation burrows, whose original depths (5–25 cm) were gradually increased during autumn and winter. By late December, frost had penetrated to  $\approx 30$  cm, but the turtles were located 1.0–1.2 m below the soil surface, where winter temperatures  $\approx 4^{\circ}\text{C}$  prevailed (M. W. Tuma, *personal communication*).

Autumnal emergence of hatchlings is the norm in northern populations of *A. spinifera* and *C. serpentina* (Ultsch 1989). Whereas successful overwintering in the nest of *A. spinifera* is probably rare (e.g., Breckenridge 1960), in *C. serpentina* it occurs at least occasionally (Bleakney 1963, Ernst 1966, Obbard and Brooks 1981, present study). Although both species succumbed in our discriminatory tests of freeze tolerance, the equivocal results for *C. serpentina* (i.e., deferred mortality after freezing at  $-2.5^{\circ}\text{C}$ ) suggest a partially developed adaptive response. Albeit limited, such capacity, which provides protection at  $T_b$ s at least as low as  $-2^{\circ}\text{C}$  (also reported for Nebraska hatchlings by Packard and Packard [1990] and Packard et al. [1993]), may be adequate

in some winters, particularly at the temperatures occurring in relatively deep *C. serpentina* nests (mean depth of the nest bottom and clutch center of  $n = 10$  nests were 20.0 and 24.9 cm, respectively). Although additional field and laboratory study is needed to assess the nature and ecological relevance of this response, freezing survival potentially explains instances of successful terrestrial overwintering since this species supercools minimally (DePari 1988) and is highly susceptible to inoculative freezing (Packard and Packard 1990, Packard et al. 1993).

*Supercooling as a cold-hardiness strategy in hatchling C. picta*

The role of supercooling as a cold-hardiness strategy is perhaps best known among the insects (e.g., Lee 1991), although this phenomenon may also promote winter survival of certain vertebrate ectotherms (for review, see Costanzo and Lee 1995). Various reptiles can supercool extensively in the laboratory with apparently little health risk (Halpern 1970, Lowe et al. 1971, Spellerberg 1972), except at very low  $T_b$  (i.e.,  $< -11^\circ\text{C}$ ; Packard and Packard 1993). Investigators earlier hypothesized that supercooling enables hatchling *C. picta* to survive subfreezing temperatures in nature (Bailey 1949, Bleakney 1963, Breitenbach et al. 1984); however, whereas some empirical evidence supports this contention (Paukstis et al. 1989, Packard and Packard 1993), other studies (e.g., Storey et al. 1988, Claussen and Zani 1991, Churchill and Storey 1992a) suggest that supercooling capacity in this species is much more limited.

Although such disparate results may partly reflect differences in experimental parameters (e.g., body size, cooling rate, duration of exposure; Costanzo and Lee 1995), they may owe largely to variation in animal hydration state. In the present study, a reduction in body water from 77 to 75% lowered the  $T_c$  of *C. picta* hatchlings from  $\approx -3^\circ$  to  $-3.4^\circ\text{C}$ . Moreover, supercooling capacity was greatest in the group kept in natural substrate over winter and permitted to dehydrate (body water = 70%). These results, considered in the context of other available data, indeed suggest a direct association between body water content and  $T_c$  in hatchling *C. picta* (Storey et al. 1988, Claussen and Zani 1991, Churchill and Storey 1992a). Notably, the highest reported  $T_c$ ,  $-1.1^\circ\text{C}$ , occurred in specimens having the highest reported body water content, 85.0% (Claussen and Zani 1991). Thus desiccation, normally considered a threat to successful overwintering (e.g., Christiansen and Gallaway 1984, Packard et al. 1989), in moderation may actually improve cold tolerance by increasing supercooling capacity. Such enhanced supercooling likely is a synergistic effect of the reduction in both tissue  $\text{FP}_{\text{eq}}$  and body fluid volume. Assuming that all osmolytes are retained during dehydration, a turtle losing 31.4% of its body water, such as in the extreme case in our study, would incur a 1.45-fold in-

crease in osmolality (e.g., from 300 to 435 mosmol/L) when solutes become concentrated in the reduced fluid volume. Given that the  $\text{FP}_{\text{eq}}$  of a 1000 mosmol/L solution is  $-1.86^\circ\text{C}$ , the  $\text{FP}_{\text{eq}}$  of turtle tissues would decrease from  $-0.56^\circ$  to  $-0.81^\circ\text{C}$ . This depression of tissue  $\text{FP}_{\text{eq}}$ , when coupled with lower tissue water content, markedly enhances organismal supercooling capacity (Costanzo and Lee 1995).

Because experimental protocols often deviate from natural cooling regimens, the results of laboratory tests of supercooling capacity must be extrapolated cautiously to field situations. Furthermore, supercooled fluids are metastable. Although the probability of spontaneous ice crystallization increases with decreasing  $T_b$ , freezing is possible at any time so long as  $T_b \leq$  tissue  $\text{FP}_{\text{eq}}$ . Thus, temporal aspects of PFEs should figure importantly in evaluations of supercooling capacity (Costanzo and Lee 1995). Low cooling rates generally favor supercooling by maximizing exposure to high temperatures and reducing the likelihood of spontaneous nucleation. Our hatchling *C. picta*, even when fully hydrated, could remain supercooled at high subzero  $T_b$ s (i.e.,  $-2^\circ\text{C}$ ) for at least several days. Under some physiological conditions, these turtles can remain unfrozen for many days at much lower  $T_b$ s (e.g., Packard and Packard 1993), which, given the extended duration of many PFEs, is a crucial facet of this species' cold hardiness.

*Susceptibility to inoculative freezing in hatchling C. picta*

Contact with environmental ice sharply limits the supercooling capacity of some animals by seeding ice nucleation within body fluids (e.g., Layne et al. 1990). Such "inoculative freezing" can also occur in reptiles when ice crystals contact mucous membranes of the cloaca, nostrils, or eyes (Lowe et al. 1971, Spellerberg 1972) or the skin (Packard et al. 1993). Some investigators (Storey et al. 1988, Packard and Packard 1993) have suggested that inoculative freezing may constrain or prevent supercooling in hatchling *C. picta* hibernating in intimate contact with the soil matrix within nests (Hartweg 1944, Breitenbach et al. 1984, DePari 1988, Packard et al. 1989).

Although our laboratory data clearly indicate that hatchling *C. picta* are susceptible to inoculative freezing, the likelihood of this event under field conditions appears contingent on microenvironmental factors, including substrate composition, water content, and moisture tension, and possibly physiological factors, such as hydration state and its influence on internal vapor pressure. Interestingly, whereas inoculative freezing occurred in all our specimens tested in damp sand (23 mg water/g dry soil), Packard and Packard (1993) observed inoculative freezing in only half of their specimens tested in a clayey soil which had a 10-fold higher water content. This disparity may reflect differences in physical properties of the experimental

substrates. Hydraulic conductivity, for example, is up to five orders of magnitude greater in sandy soils than in clayey soils (Hillel 1971). Although water potential may also be an important factor, moisture tension was comparable in our sand ( $-72$  kPa) and the clay ( $-50$  kPa) used by Packard and Packard (1993). Nevertheless, because sandy soils predominate at our study area (and in many areas where *C. picta* nests), freezing risk appears high so long as the soil matrix within *C. picta* nests remains damp.

During mid-October 1990, following several months of near normal precipitation, the water content of soil within natal nests was  $16.6$  mg/g ( $-725$  kPa). Unfortunately, we cannot discern from our experimental data (Table 5) whether this moisture level would promote inoculative freezing in hatchling *C. picta*. However, soil moisture levels in mid-October 1993 were fourfold higher ( $67.5$  mg/g;  $>-50$  kPa), doubtless owing to the abundant rain of the preceding months. Turtles thus are subject to inoculative freezing at least in wet years, unless the soil dries markedly prior to freezing. Fortunately, soil in *C. picta* nest chambers is porous and drains quickly, and relatively little rainfall (e.g.,  $7.3$  cm on average) occurs during October–February, the coldest months of hibernation. Whether increased hatchling mortality due to freezing occurs during particularly wet winters (such as that of 1993–1994) is unknown. Additional research is needed to assess fully the roles of physiological and environmental conditions influencing inoculative freezing.

*Winter survival strategy of hatchling  
C. picta: supercooling or  
freeze tolerance?*

Survival of hatchling *C. picta* exposed to subzero temperatures may be promoted by two mechanisms, freeze tolerance or supercooling, each of which has specific limitations. Freeze tolerance permits survival of frozen tissues but protects turtles only at high subzero  $T_{b,s}$  (e.g.,  $\geq -4^{\circ}\text{C}$ ; Storey et al. 1988, Claussen and Zani 1991, Churchill and Storey 1992a). Supercooling permits survival at much lower  $T_{b,s}$  (e.g., to  $\approx -12^{\circ}\text{C}$ ; Packard and Packard 1993), but the efficacy of this mechanism hinges on prevailing physiological and microenvironmental conditions, such as the animal's hydration state, substrate characteristics, and the presence of environmental ice.

Collectively, our laboratory and field data indicate that the cold-hardiness strategy of hatchling *C. picta* may be predicated on both supercooling and freeze tolerance capacities, and further suggest a model elucidating the role of each mechanism under specific microenvironmental and physiological conditions. Accordingly, supercooling would predominate during periods of low environmental water potential, since drying of the soil matrix promotes desiccation of hatchlings within the nest. Supercooling capacity would thus increase, permitting turtles to survive even

protracted exposure to very low temperatures with minimal risk of freezing. Conversely, during exposure to damp soil, such as after periods of rain or snow melt, hatchlings would rehydrate, relinquish supercooling capacity, and incur an increased risk of freezing. In this scenario, the susceptibility of *C. picta* hatchlings to inoculative freezing (which likely eliminates supercooling altogether) is actually beneficial, given that spontaneous crystallization of deeply supercooled body fluids promotes injury or death owing to the attendant surge in ice formation (Claussen and Costanzo 1990, Costanzo and Lee 1995). Freezing survival would also be promoted by the heat liberated by freezing soil, which moderates the rate of tissue freezing, a critical determinant of freezing survival (Costanzo and Lee 1995). Ultimately, however, cooling of the frozen turtle below  $-3^{\circ}$  to  $-4^{\circ}\text{C}$  would prove lethal.

The distinct levels of thermal protection conferred by these survival mechanisms may account for the reported differences in winter survival of hatchling *C. picta* (Table 6). For example, whereas hatchlings in some locales tolerate winter temperatures  $<-10^{\circ}\text{C}$  (Woolverton 1963, DePari 1988), in others temperatures as high as  $-5^{\circ}\text{C}$  may be lethal (e.g., St. Clair and Gregory 1990). Low winter survival of some northern *C. picta* (e.g., Breitenbach et al. 1984, St. Clair and Gregory 1990, Lindeman 1991) may reflect regionally higher risks of freezing associated with poorly drained soils. Interestingly, clayey soils, although they may provide ideal conditions for embryonic development (e.g., Christens and Bider 1987), may be a liability during overwintering, particularly when insulation in the form of snow cover is lacking.

Generally, supercooling and freeze tolerance are dichotomous strategies for coping with subzero temperatures (Lee 1991, Costanzo and Lee 1995). However, some coleopterans and several species of nematodes switch between these modes (e.g., Block 1991). A cold-hardiness strategy based on both survival mechanisms, as originally suggested by Paukstis et al. (1989) and Ultsch (1989), may promote winter survival in hatchling *C. picta* by conferring protection under dynamic physiological and microenvironmental conditions. Definitive support for such an hypothesis, presumably obtained through the careful monitoring of hibernating individuals, would reconcile the conflicting hypotheses of current investigators (Storey et al. 1988, Packard and Packard 1993a).

*Overwintering strategies influence  
turtle demography*

Patterns of hatchling emergence behavior are correlated with morphological adaptations for burrowing and physiological cold hardiness, factors that may limit the northern range of some turtles. The occurrence of suitably friable soils in the north-central United States and the coupling of burrowing behavior, promoted by a progressive shortening of the phalanges and reduction

of interdigital webbing (Legler 1960), and freeze tolerance, likely has permitted *T. ornata* to range farther north in North America than other terrestrial turtles. Endowed with enlarged forelimbs and heavy foreclaws, *K. flavescens* is also morphologically adapted to fossorial activity, but clearly lacks freeze tolerance, both as hatchlings and as adults. Hence, the northernmost populations of this species are limited to areas where loose soil facilitates burrowing below the frost line (e.g., Christiansen and Bickham 1989, Iverson 1991b). Apparently the costs of such activity, including the energy investment of finite energy reserves and retarded spring emergence, are offset by reduced winter mortality.

By virtue of their morphology, hatchlings of the aquatic *C. serpentina* and *A. spinifera* are not only ill suited for digging, but are also vulnerable to dehydration (e.g., Christiansen and Gallaway 1984) and thus usually emerge from natal nests in autumn and hibernate under water (Ultsch 1989). The northern distributions of both *C. serpentina* and *A. spinifera* are at least partly constrained by the length of the activity season, since eggs must be laid early enough in summer so that hatching and nest emergence can occur before winter. Enigmatically, hatchling *C. picta* overwinter within the nest, even at the northern limit of the species' range. However, its unique cold-hardiness strategy has permitted this species to colonize habitats farther north than most North American turtles.

Our results also suggest a phylogenetic aspect to cold hardiness in turtles. Of the four turtle families represented in this study, members of only one (the Emydidae) proved to be freeze tolerant. This relationship is bolstered by evidence for a limited freeze tolerance in *Trachemys scripta* (Churchill and Storey 1992b). Given that the center of diversity of this turtle family is at a higher latitude than that of any other (Iverson 1992), it is possible that freeze tolerance has evolved only once in the Testudines. However, verification of this conclusion must await further studies of freeze tolerance in other species both within and outside the Emydidae.

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