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# Cold-Hardiness and Evaporative Water Loss in Hatchling Turtles

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## ABSTRACT

North American turtles hatch in late summer and spend their first winter either on land or underwater. Adaptations for terrestrial overwintering of hatchlings in northern regions, where winter thermal and hydric regimes are harsh, have not been systematically investigated in many species. We measured intrinsic supercooling capacity, resistance to inoculative freezing, and desiccation resistance in hatchlings of terrestrial and aquatic turtles collected from northern (*Terrapene ornata*, *Chrysemys picta bellii*, *Kinosternon flavescens*, *Chelydra serpentina*) and southern (*Chrysemys picta dorsalis*, *Trachemys scripta*, *Sternotherus odoratus*, *Sternotherus carinatus*) locales. Supercooling capacity was estimated from the crystallization temperature of turtles cooled in the absence of external ice nuclei. Mean values ranged from −8.1° to −15.5°C and tended to be lower in terrestrial hibernators. Inoculation resistance was estimated from the crystallization temperature of turtles cooled in a matrix of frozen soil. These values (range of means: −0.8° to −13.6°C) also tended to be lower in the terrestrial hibernators, especially *C. picta bellii*. Mean rates of evaporative water loss varied markedly among the species (0.9–11.4 mg $g^{-1}$ $d^{-1}$) and were lowest in the terrestrial hibernators. Most species tolerated the loss of a modest amount of body water, although half of the sample of *S. carinatus* died from desiccation. In general, turtles did not regain lost body water from wet soil, and immersion in free water was required for rehydration. Therefore, desiccation resistance may be an important adaptation to terrestrial hibernation. Resistances to inoculative freezing and desiccation were directly correlated, perhaps because they are governed by the same morphological characteristics.

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## Introduction

North American turtles commonly hatch in late summer or early autumn and then overwinter either on land or underwater. Remaining on land during winter is thought to reduce the risk of predation and conserve endogenous fuel reserves at a time when food resources are scarce (Gibbons and Nelson 1978). Nevertheless, in northern regions, hatchlings of many species overwinter in aquatic habitats, which tend to be more thermally buffered than terrestrial ones (Ultsch 1989). At least a few species hibernate on land in cold climates, although little is known about their physiological and ecological adaptations for winter survival. Such adaptations may include burrowing to evade frost, freeze avoidance through supercooling, and freeze tolerance.

Our initial investigation of the overwintering biology of northern turtles focused on an assemblage of species endemic to the Sandhills region of west-central Nebraska (Costanzo et al. 1995). There, hatchlings of the western painted turtle (*Chrysemys picta bellii*) hibernate within their natal nests, 8–12 cm below the ground surface, and neonatal ornate box turtles (*Terrapene ornata*) and yellow mud turtles (*Kinosternon flavescens*) overwinter 1 m or more beneath the floor of the nest chamber. In contrast, hatchlings of the spiny softshell turtle (*Apalone spinifera*) and common snapping turtle (*Chelydra serpentina*) emerge from their nests shortly after hatching and move to ponds or streams for hibernation. Differences in the overwintering habits of these turtles likely reflect variation in adaptations to fossorialism and cold-hardiness (Costanzo et al. 1995). We suspect that choice of winter habitat may also be influenced by the ability to resist desiccation.

In general, supercooling capacity is influenced by body size, the quantity and distribution of water within the body, and the osmotic pressure of body fluids (see review in Lee and Costanzo 1998), but whether these factors also influence supercooling in hatchling turtles has not been tested. Supercooling capacity may be constrained by environmental ice nuclei that seed the freezing of body fluids (Lee and Costanzo 1998); therefore, species likely vary in their ability to remain supercooled under field conditions. Such inoculative freezing of hatchlings in natal nests is promoted by physical contact with ice in the soil matrix (Packard and Packard 1993*b*; Costanzo et al. 1998) as well as with other ice nuclei that commonly occur in nesting soils (Costanzo et al. 2000*a*). Resistance to inoculative freezing has been studied in few species, and results from different inves-

tigations are not always comparable. We hypothesize that inoculation resistance is particularly well developed in species whose hatchlings hibernate on land.

Dehydration may be a significant mortality factor among terrestrially overwintering reptiles (Gregory 1982). Turtles hibernating within the frost zone may be subjected to chronic water stress because precipitation in winter may fall as snow rather than as rain and because water potential within a frozen soil matrix is low. Morphological and physiological mechanisms of water conservation thus may be important to terrestrial overwintering, especially if turtles are unable to rehydrate within their hibernacula. Few measurements of evaporative water loss (EWL) have been made for adult turtles (Stone and Iverson 1999), and we have found no data for hatchlings.

Our purpose was to compare supercooling capacity, resistance to inoculative freezing, and rates of EWL among hatchlings of species adapted to habitats ranging from terrestrial to aquatic and of species that hibernate on land or underwater. We studied *C. picta bellii*, *K. flavescens*, *T. ornata*, and *C. serpentina* indigenous to our study site in Nebraska. We also studied hatchlings of species that inhabit more southern locales, including the southern painted turtle (*Chrysemys picta dorsalis*), red-eared slider (*Trachemys scripta*), stinkpot (*Sternotherus odoratus*), and razor-backed musk turtle (*Sternotherus carinatus*). Both *C. picta dorsalis* and *T. scripta* apparently overwinter on land as hatchlings (see Cagle 1950, 1954; J. Iverson, unpublished observation), whereas *S. odoratus* and *S. carinatus* probably hibernate underwater (Nagle et al. 1998; but see Gibbons and Nelson 1978). Our hypothesis is that species that hibernate on land in cold climates exhibit greater capacities for supercooling and inoculation resistance and resist desiccation better than do species that hibernate underwater in cold climates and species that are restricted to southern regions.

## Material and Methods

### *Source of Animals*

Eggs of eight taxa of turtles were collected in summer 1997 and/or 1998 from oxytocin-treated females, transported to the laboratory, and incubated to hatching at approximately 29°C in moist vermiculite (1.0 g water $g^{-1}$ dry vermiculite; approximately −150 kPa). We obtained eggs of western painted turtles (*Chrysemys picta bellii*), yellow mud turtles (*Kinosternon flavescens*), common snapping turtles (*Chelydra serpentina*), and ornate box turtles (*Terrapene ornata*) near Gimlet Lake, Crescent Lake National Wildlife Refuge, Garden County (41°N, 102°W), west-central Nebraska. Eggs of southern painted turtles (*Chrysemys picta dorsalis*), red-eared sliders (*Trachemys scripta*), and stinkpots (*Sternotherus odoratus*) were obtained in Lonoke County (34°N, 55°W), central Arkansas, and eggs of razor-backed musk turtles (*Sternotherus carinatus*) were collected in McCurtain County (34°N, 54°W), extreme southeastern Oklahoma.

The eggs used in this investigation were collected for use in several projects. Those allocated to this study were randomly selected from a pool of available material created by combining clutches obtained from the number of females indicated as follows: *T. ornata*, five; *K. flavescens*, five; *T. scripta*, six; *C. picta dorsalis*, two; *S. odoratus*, 28; and *S. carinatus*, 20. For *C. picta bellii* and *C. serpentina*, the exact number of females from which eggs were obtained was not determined, but in each case the number was >20.

On hatching, in late August to mid-September, turtles were transferred to darkened plastic boxes containing damp vermiculite (0.5 g water $g^{-1}$ dry vermiculite; approximately −350 kPa) and held at 22°C to simulate conditions within the nests in late summer. We then acclimated the turtles to winter temperatures by gradually reducing ambient temperature. Turtles from Nebraska were placed in an environmental chamber set at 15°C on October 1. After 30 d, the temperature was lowered and the turtles were kept at 10°C for 30 d. On December 1, the temperature was reduced a final time, and turtles were thereafter kept at 5°C. Cold acclimation of the turtles from southern locales was initiated on October 15 but otherwise followed the same regimen. Turtles were selected at random from their holding boxes and used in experiments in midwinter.

### *Supercooling and Inoculative Freezing Trials*

We measured supercooling capacity by cooling turtles in the absence of external ice nuclei until they spontaneously froze (Costanzo et al. 1998). Turtles ($n$ = 3–8 per taxon) were prepared for testing by gently brushing vermiculite from their surfaces and then holding them for 24 h at 5°C in darkness in sheltered cups. This procedure permitted evaporation of surface moisture, which otherwise might have frozen and inoculated turtle tissues. Each turtle was instrumented with a 30-gauge thermocouple (copper-constantan) attached to the carapace using quick-setting epoxy, placed individually in a 50-mL plastic tube, and insulated by loosely filling the superjacent space with plastic foam. The tubes were then suspended in a refrigerated ethanol bath (Neslab, model RTE 140, Portsmouth, N.H.). Turtles were held at −0.4°C for 1 h before being further cooled (0.5°C $h^{-1}$) until each produced a freezing exotherm. During cooling, turtle temperature, as registered by the thermocouple, was logged at 30-s intervals on a data logger (Omega model RD3752, Stamford, Conn.). The temperature of crystallization ($T_c$) of each hatchling was determined from the recording.

Inoculative freezing trials were conducted by cooling turtles ($n$ = 4–9 per taxon) in a matrix of frozen substratum. Hatchlings were prepared for testing as described above and placed individually in 50-mL plastic tubes containing approximately 12 g of substratum (see below). To ensure that turtles were uniformly exposed to ice nuclei, a sufficient quantity of sub-

stratum was used to achieve a substratum : body mass ratio of 4 : 1, and the material was firmly tamped around the animal. The space above the substratum was then filled with a piece of plastic foam. Turtles were habituated to the substratum for 24 h at 5°C in darkness, and trials were then initiated by suspending the tubes in the refrigerated bath. After the turtles attained thermoequilibrium at −0.4°C (a temperature at which the soil, but not the turtles, could freeze), the substratum was inoculated with small ice crystals and permitted to freeze for 1 h. Subsequently, the temperature inside the tube was reduced at 0.5°C $h^{-1}$ until each turtle produced a freezing exotherm and the $T_c$ of the turtle was determined as in the supercooling trials.

The substratum used in these trials was a composite of soil samples collected at our field site in September 1996 (see Costanzo et al. 1998). Soil was sampled at a depth of 10 cm from seven locales, each <0.5 m from a *C. picta* nest constructed the previous year. It was stored at 4°C in covered containers and combined in equal quantities before use. The pooled material (loamy sand) was sieved with a 2-$mm^2$ mesh, mixed (9 : 1) with fine clay (see Costanzo et al. 1998), and then autoclaved to destroy any organic ice nuclei. Finally, it was dried thoroughly at 65°C and then hydrated (0.075 g water $g^{-1}$ dry mass; water potential = −400 kPa) with autoclaved, ultrapurified water.

### *Evaporative Water Loss*

Turtles ($n$ = 3–10 per taxon) were removed from their holding boxes, brushed free of adherent vermiculite, and weighed on a balance (Mettler-Toledo, model AG245, Hightstown, N.J.) to the nearest 0.1 mg. They were confined to stalls (6 × 8 cm), fashioned from plastic screens, inside a plastic box that served as a desiccation chamber. The box was continuously ventilated with cold air (5°C, 75%–80% RH) at a rate that replaced the air volume approximately 2.5 times $h^{-1}$.

We weighed turtles at intervals of approximately 24 h over the next 10 d and calculated EWL from the decrease in body mass. However, data from the first 24 h and last 48 h were omitted from the calculation because in some species the change in body mass was nonuniform during these periods. To assess their tolerance to desiccation, we determined before each weighing whether turtles would respond to being prodded with a blunt probe.

### *Rehydration Experiment*

At the conclusion of the EWL trials, groups of three to five turtles of mixed species were immersed in water-saturated sand and held for 10 d at 5°C in darkness. Subsequently, the turtles were gently brushed to remove as much adherent sand as possible, dried of surface moisture by holding them for 24 h at 5°C in sheltered cups, and reweighed to the nearest 0.1 mg. The change in body mass was used to ascertain whether turtles had absorbed moisture from the substratum.

Next, we determined whether the same turtles could rehydrate during immersion in water. Turtles were kept in a shallow pool of water (5°C, in darkness) for 8 d and then blotted with laboratory tissue, air dried for 24 h (as before) to remove surface moisture, and reweighed for a final time. Turtles from this experiment were killed by freezing them at −80°C, thawed, used in morphometric determinations (see below), and then dried to constant mass in a 65°C oven. The percentage body water at the outset and at the end of the EWL experiment and after each phase of the rehydration experiment was calculated on the assumption that dry body mass remained constant.

### *Morphometric Analyses*

We conducted several analyses to determine how morphological variation influenced cold-hardiness and desiccation resistance in hatchling turtles. These analyses were based on our beliefs that environmental ice nuclei are transmitted across the skin or soft tissues but, apparently, not the shell (Packard and Packard 1993*a*; Packard et al. 1993, 1997) and that EWL is impeded by the shell and occurs primarily across the skin (Rose 1969; Spotila and Berman 1976). The exposed skin surface is a critical element in the processes under investigation. However, because hatchlings likely reduce skin exposure to ice nuclei and dry air by retracting (as much as possible) the head and limbs within the shell (Packard and Packard 1995), measuring the entire cutaneous surface area (e.g., Stone and Iverson 1999) would overestimate the functional exchange surface. Rather, our analyses focused on the critical anatomical regions; namely, the openings to the limb and nuchal pockets and any skin on the ventral surface that is not covered by the shell.

We photocopied the ventral aspect of turtles arranged in a natural posture (i.e., head and limbs partially retracted) and then scanned (AGFA Duoscan, using Adobe Fotolook 32, v2.09.04) the images for use with analytical software (Image-Pro Plus). We traced the margin of the plastron and contiguous lateral bridges to determine the area of these structures. We then traced the exposed margin of the (larger) carapace, carefully interpolating short segments of the tracing where view of the margin was obscured by a protruding head or limb. Subtracting the plastral area from the carapacial area gave an estimate of critical exposure area, the two-dimensional surface at openings to the limb and nuchal pockets. We also computed a size-independent critical exposure index as the ratio of carapacial area to plastral area. This index is low in species with expansive plastrons and high in species with much-exposed skin on the ventral body surface.

*Statistical Analysis*

In most experiments, sample means were compared using ANOVA or ANCOVA with body mass or percentage body water as a covariate; multiple comparisons were made using Student-Newman-Keuls (SNK) tests. In the rehydration experiment, repeated-measure ANOVAs, followed by Bonferroni tests, were used to analyze changes in body mass. Simple regression analyses were used to examine relationships between morphometric measures and EWL or resistance to inoculative freezing. Mean values are presented ±1 SE. Differences were considered significant at $P < 0.05$.

## Results

*Supercooling Capacity and Resistance to Inoculative Freezing*

All species supercooled when chilled in the absence of environmental ice nuclei (Table 1). However, the mean temperature of crystallization ($T_c$) varied ($F_{6,38} = 6.9$, $P < 0.0001$) among the species. The lowest $T_c$, −17.8°C, was recorded for a hatchling *Chrysemys picta bellii*, a species in which supercooling was especially well developed. We found marked interspecific variation in two factors that potentially influence supercooling capacity: body mass ($F_{6,38} = 231.9$, $P < 0.0001$) and percentage body water ($F_{6,38} = 33.1$, $P < 0.0001$). However, neither body mass ($F_{1,36} = 0.6$, $P = 0.44$) nor percentage body water ($F_{1,36} = 1.1$, $P = 0.31$) covaried with $T_c$ in the analyses.

Mean $T_c$ values for turtles chilled in contact with frozen substratum varied among the species ($F_{7,48} = 25.9$, $P < 0.0001$), but only *C. picta bellii* supercooled to very low temperatures (Table 1). Indeed, the mean values of $T_c$ for *C. picta bellii* cooled in the absence or presence of environmental ice nuclei were similar ($t = 1.09$, df = 11, $P = 0.29$; Table 1), indicating that this species fully resisted inoculative freezing. Although mean values of $T_c$ among the other species did not differ ($P > 0.05$), a scatterplot of the values for individual turtles suggested that the propensity to resist inoculative freezing was greater in terrestrial hibernators than in aquatic hibernators (Fig. 1). For example, whereas some *Kinosternon flavescens* and *Trachemys scripta* supercooled to −6°C or below, most *Chelydra serpentina*, *Sternotherus odoratus*, and *Sternotherus carinatus* froze at temperatures very near the equilibrium melting point of their tissues, approximately −0.6°C. Accordingly, there was less variation in the data for aquatic hibernators (equality of variances *F*-test, $P < 0.05$).

*Evaporative Water Loss*

Turtles lost between 0.7% (*Terrapene ornata*) and 8.7% (*S. carinatus*) of their initial body mass during the EWL trials. Rates of EWL varied ($F_{7,46} = 36.4$, $P < 0.0001$; Table 2) among the species, although body mass was a significant covariate in this analysis (ANCOVA; $F_{1,45} = 5.2$, $P = 0.028$). Dividing the EWL rate by the body mass of individual turtles effectively eliminated mass as a covariate ($F_{1,45} = 0.1$, $P = 0.82$). Comparisons of these mass-adjusted values again showed that EWL varied among species ($F_{7,46} = 32.9$, $P < 0.0001$) and tended to be higher in aquatic turtles. Notably, *C. picta bellii* lost water at a rate typical of more terrestrial species (Table 2).

Most turtles were responsive to tactile stimulation and ultimately survived the EWL experiments. However, two *S. carinatus* died during the final day, and a third died during the subsequent rehydration experiment, presumably as a result of desiccation. This species had a comparatively low percentage body water at the outset of the EWL experiment (Table 2).

Table 1: Supercooling capacity and resistance to inoculative freezing, as indicated by the temperature of crystallization ($T_c$) of turtles immersed in frozen substratum in hatchling turtles that overwinter on land or underwater

| Species | Hibernation Habitat | $T_c$ (°C) | |
|---|---|---|---|
| | | Supercooling (*N*) | Inoculation (*N*) |
| *Terrapene ornata* | Terrestrial | −12.0 ± 1.6$^{A,C}$ (3) | −2.4 ± .5$^{A}$ (8) |
| *Kinosternon flavescens* | Terrestrial | −9.3 ± 1.5$^{C}$ (5) | −3.1 ± 1.1$^{A}$ (7) |
| *Trachemys scripta* | Terrestrial | −13.9 ± 1.4$^{A,B}$ (8) | −3.0 ± 1.1$^{A}$ (8) |
| *Chrysemys picta bellii* | Terrestrial | −15.5 ± 1.2$^{A}$ (8) | −13.6 ± .9$^{B}$ (5) |
| *Chrysemys picta dorsalis* | Terrestrial | nd (0) | −2.5 ± .8$^{A}$ (4) |
| *Chelydra serpentina* | Aquatic | −8.5 ± .5$^{C}$ (8) | −1.5 ± .4$^{A}$ (9) |
| *Sternotherus odoratus* | Aquatic | −8.1 ± 1.1$^{C}$ (8) | −1.3 ± .3$^{A}$ (9) |
| *Sternotherus carinatus* | Aquatic | −10.6 ± .7$^{B,C}$ (5) | −.8 ± .1$^{A}$ (6) |

Note. Means (±SE) identified by common letter were not statistically distinguishable (ANOVA, Student-Newman-Keuls multiple-comparisons; $P < 0.05$); nd = not determined.

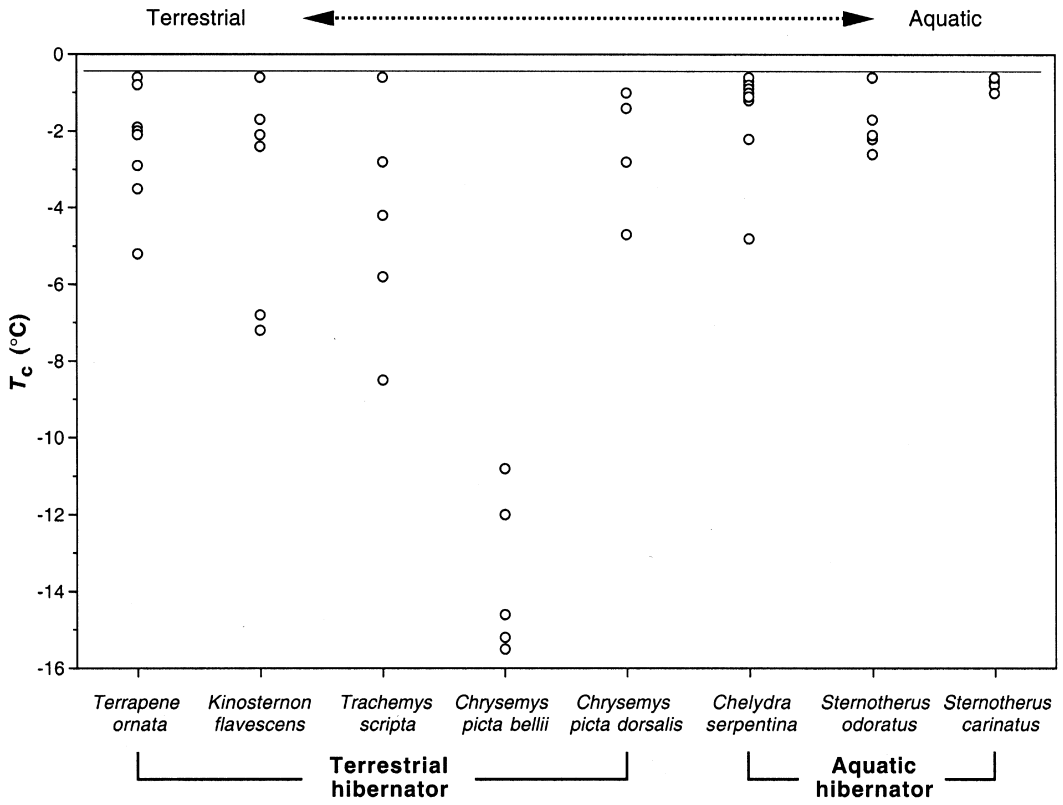


Figure 1. Resistance to inoculative freezing in hatchlings of eight turtle taxa, as indicated by the temperature of crystallization ($T_c$) of turtles immersed in frozen substratum. Each point represents an individual hatchling; sample sizes are as given in Table 1. Horizontal line represents the approximate equilibrium freezing point of turtle tissues.

*Rehydration of Turtles in Wet Substratum and Free Water*

Mean body mass increased nominally in several species during submersion in wet sand (Fig. 2). However, the increase was statistically significant (Bonferroni: $t = 4.90$; $P < 0.05$) only for *T. ornata*, which regained 45%–96% of the small amount of body mass lost during the EWL experiments. Body mass increased by 63–258 mg in *S. carinatus*, but the change was not significant (Bonferroni: $t = 2.92$; $P > 0.05$), probably because of the small size of the sample. Results for *S. odoratus* were equivocal: some specimens apparently gained as much as 202 mg, whereas others lost as much as 40 mg (Fig. 2). We had presumed that any increase in body mass reflected only absorption of water from the soil; however, despite our efforts to thoroughly clean the turtles, soil particles may have lodged in their axillary pockets and skin folds, confounding interpretation of the results.

Turtles immersed in free water generally gained body mass (Fig. 2). Most terrestrial hibernators (*T. ornata*, *C. picta bellii*, and *C. picta dorsalis*) returned to their respective predesiccation body masses, although *K. flavescens* overshot this mark by approximately 4.5% and *T. scripta* showed no change in body mass after water immersion (Fig. 2). The aquatic hibernators (*S. odoratus*, *S. carinatus*, and *C. serpentina*) weighed about 5% less than they did before desiccation and, thus, did not fully rehydrate.

*Somatic and Morphological Measurements*

Body mass varied ($F_{7,46} = 117.0$, $P < 0.0001$) among the eight taxa used in the EWL/rehydration experiments, as did initial percentage body water ($F_{7,46} = 19.8$, $P < 0.0001$; Table 2). Mean percentage body water was directly correlated with dry body mass ($F_{1,52} = 9.9$, $P = 0.003$, $r^2 = 0.16$) but showed no association with hibernation habitat (Tables 1, 2).

Estimates of critical exposure area varied among the species ($F_{7,46} = 81.3$, $P < 0.0001$), being lowest for *K. flavescens* and *C. picta bellii* and highest for *C. serpentina* (Table 2). Not unexpectedly, body mass was a significant covariate ($F_{1,45} = 17.8$, $P = 0.0001$) in the analysis, with larger turtles generally having greater critical exposure area irrespective of shell morphology.

Table 2: Evaporative water loss (EWL) and morphometric variables in hatchlings of eight turtle taxa

| Species | *N* | Body Mass (g) | Percentage Body Water | EWL: Percentage Initial Mass | EWL: mg $h^{-1}$ | EWL: mg $g^{-1}$ $d^{-1}$ | Critical Exposure Area ($mm^2$) | Critical Exposure Index |
|---|---|---|---|---|---|---|---|---|
| Terrestrial hibernator: | | | | | | | | |
| *Terrapene ornata* | 4 | 10.7 ± .3$^{A}$ | 79.0 ± .6$^{A}$ | .7 ± .03$^{A}$ | .41 ± .03$^{A}$ | .91 ± .04$^{A}$ | 300 ± 19$^{A}$ | 1.39 ± .03$^{A}$ |
| *Kinosternon flavescens* | 6 | 3.1 ± .1$^{B}$ | 79.5 ± .4$^{A}$ | 1.7 ± .1$^{A}$ | .31 ± .02$^{A}$ | 2.38 ± .15$^{A}$ | 147 ± 7$^{B}$ | 1.65 ± .02$^{A}$ |
| *Trachemys scripta* | 10 | 7.7 ± .4$^{C}$ | 80.6 ± .3$^{A}$ | 1.8 ± .1$^{A}$ | .81 ± .07$^{A}$ | 2.61 ± .14$^{A}$ | 372 ± 15$^{C}$ | 1.65 ± .01$^{A}$ |
| *Chrysemys picta bellii* | 6 | 4.0 ± .1$^{D}$ | 79.4 ± .6$^{A}$ | 1.4 ± .2$^{A}$ | .32 ± .05$^{A}$ | 1.90 ± .25$^{A}$ | 160 ± 8$^{B}$ | 1.44 ± .02$^{A}$ |
| *Chrysemys picta dorsalis* | 3 | 5.0 ± .1$^{E}$ | 79.5 ± .8$^{A}$ | 2.6 ± .02$^{A}$ | .76 ± .03$^{A}$ | 3.61 ± .02$^{A}$ | 274 ± 6$^{A}$ | 1.61 ± .01$^{A}$ |
| Aquatic hibernator: | | | | | | | | |
| *Chelydra serpentina* | 10 | 8.7 ± .2 | 82.5 ± .3$^{B}$ | 4.7 ± .3$^{B}$ | 2.27 ± .13$^{B}$ | 6.28 ± .34$^{B}$ | 583 ± 15$^{D}$ | 3.54 ± .09$^{B}$ |
| *Sternotherus odoratus* | 9 | 3.2 ± .1$^{B}$ | 79.5 ± .4$^{A}$ | 7.2 ± 1.0$^{C}$ | 1.28 ± .19$^{C}$ | 9.61 ± 1.28$^{C}$ | 252 ± 9$^{A}$ | 2.48 ± .07$^{C}$ |
| *Sternotherus carinatus* | 6 | 2.8 ± .1$^{B}$ | 75.8 ± .7$^{C}$ | 8.7 ± .2$^{C}$ | 1.34 ± .02$^{C}$ | 11.37 ± .04$^{C}$ | 250 ± 15$^{A}$ | 2.50 ± .07$^{C}$ |

Note. Values are means ± SE. Within each column, means identified by common letter were not statistically distinguishable (ANOVA, Student-Newman-Keuls multiple-comparisons; $P < 0.05$).

Critical exposure index, a mass-independent ($F_{1,45} = 1.6$, $P = 0.21$) measure of the ventral surface unprotected by shell, also varied by species ($F_{7,46} = 147.0$, $P < 0.0001$) and ranged from 1.4 in *T. ornata* to 3.5 in *C. serpentina* (Table 2).

Species having high estimated critical exposure areas generally exhibited high rates of EWL (Table 3). Also, critical exposure index and mass-adjusted rates of EWL were weakly correlated. We found a positive correlation approaching statistical significance between critical exposure index and degree of inoculation resistance. However, the values of $T_c$ for turtles cooled in contact with frozen substratum were not correlated with critical exposure area (Table 3).

## Discussion

### *Supercooling Capacity and Inoculation Resistance in Hatchling Turtles*

In general, supercooling capacity is inversely related to body size and body water content (see review by Lee and Costanzo 1998). However, our results suggest that large body size (up to 10.7 g in *Terrapene ornata*) and relatively high body water content (up to 82.5% in *Chelydra serpentina*) do not preclude the use of supercooling as a winter survival mechanism in hatchling turtles. All species supercooled to −8°C or below, but whether or not they can survive chilling at such temperatures is unknown because our trials culminated in spontaneous freezing and death. However, *Chrysemys picta bellii* recovers from supercooling to temperatures as low as −10° to −15°C (Costanzo et al. 1999; Packard and Packard 1999), and *C. serpentina* tolerates supercooling to at least −5°C (Packard et al. 1993; Costanzo et al. 1999).

Supercooling capacities of our turtles tended to reflect the thermal extremes encountered by each species within their particular winter microenvironment. For example, the terrestrial hibernators—*T. ornata*, *Trachemys scripta*, and (especially) *C. picta bellii*—supercooled to low temperatures, whereas the aquatic hibernators—*C. serpentina*, *Sternotherus odoratus*, and *Sternotherus carinatus*—supercooled less extensively. Supercooling capacity was also less developed in *Kinosteron flavescens*, a species that hibernates deep in the soil, below the reach of frost (Costanzo et al. 1995).

What accounts for interspecific variation in supercooling capacity among hatchling turtles? Costanzo et al (2000*b*) found that seasonal development of supercooling capacity in hatchling *C. picta bellii*, but not *C. serpentina*, involves elimination (or attenuation) of endogenous ice nuclei and a marked increase in plasma osmotic pressure. Because of a limited availability of specimens, we were unable to investigate supercooling capacity in the more southerly distributed subspecies, *Chrysemys picta dorsalis*, which presumably hibernates terrestrially. Such a comparison would have enabled us to determine whether northward expansion of the species range, following glacial retreat (Bleakney 1958), was accompanied by an adaptive increase in innate supercooling capacity.

Because terrestrially hibernating turtles are commonly exposed to ice and other environmental ice nuclei that may seed the freezing of their body fluids, an intrinsic capacity for supercooling is of little value unless the animal can resist inoculative freezing. Studies of inoculation resistance in *C. picta bellii* (Packard and Packard 1993*b*; Costanzo et al. 1998, 2000*c*), *C. serpentina* (Packard et al. 1993), and *T. scripta* (Packard et al. 1997) bolster our finding that inoculation resistance is better developed in terrestrial hibernators. In particular, the resistance exhibited by *C. picta bellii* is exceptional, especially when considered in relation to that of *C. picta dorsalis* (Fig. 1).

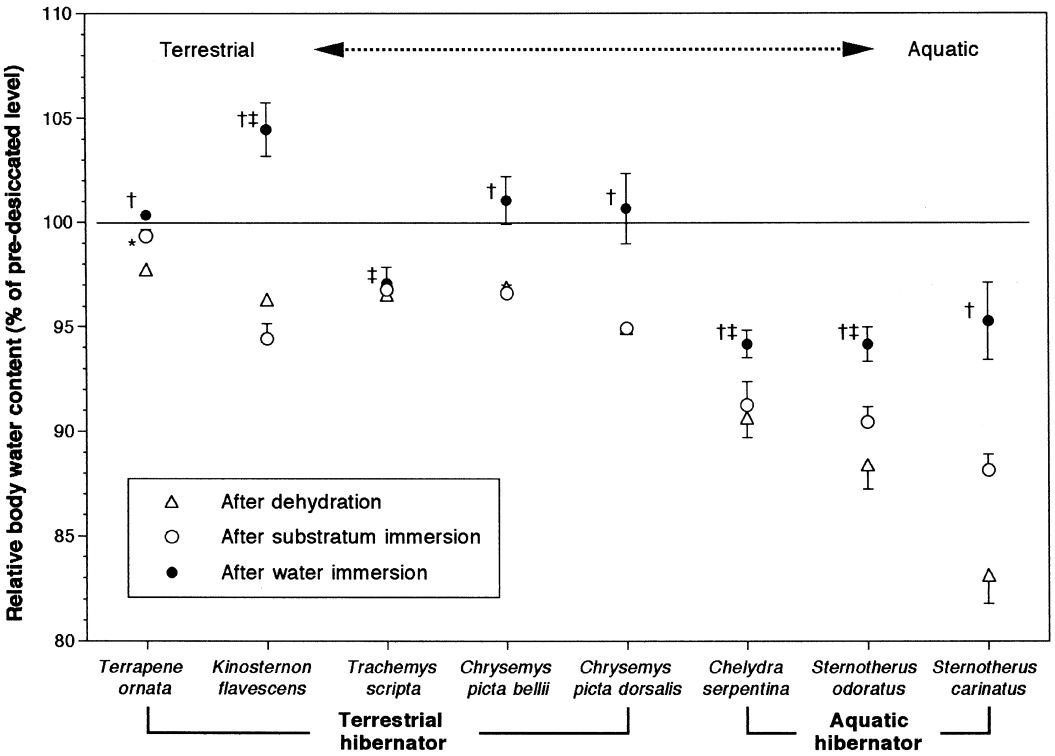


Figure 2. Mean (±SE) relative body water content of turtles determined sequentially after dehydration, immersion in wet substratum, and immersion in water. An asterisk indicates that the mean for turtles after substratum immersion differed from the corresponding value after desiccation; a dagger indicates that the mean for turtles after water immersion differed from the corresponding value after substratum immersion; and a double dagger indicates that the mean for turtles after water immersion differed from the corresponding value before dehydration, which is represented by the horizontal line. Sample sizes are as given in Table 2. (Repeated-measures ANOVA; Bonferroni, $P < 0.05$).

*Cold-Hardiness and Winter Habitat Selection*

Our results reveal interesting associations between cold-hardiness and winter habitat selection among hatchling turtles. Species that hibernate underwater, such as *C. serpentina*, *S. odoratus*, and *S. carinatus*, lack both inoculation resistance and freeze tolerance (Table 1; Fig. 1; see also Costanzo et al. 1995; Packard et al. 1999). *Kinosternon flavescens* was moderately resistant to inoculative freezing, but this freeze-intolerant species hibernates in deep underground burrows (Costanzo et al. 1995). The limited resistance exhibited by hatchling *T. scripta*, which overwinter within the natal nest but reportedly (Packard et al. 1999) lack freeze tolerance, supports the contention that the northern extent of this species' range is limited by frost penetration of the soil (Packard et al. 1997). The freeze-tolerance status of *C. picta dorsalis* has not been determined; however, given its southerly distribution, neither freeze tolerance, nor resistance to inoculative freezing, would be of consequence to winter survival.

Although inoculative freezing has deleterious consequences for many animals, it may actually benefit freeze-tolerant species by inducing somatic freezing at the high temperatures conducive to freezing survival (Lee and Costanzo 1998). Low resistance to inoculative freezing may be adaptive in *T. ornata* because this freeze-tolerant species hibernates below the nest, potentially within the reach of frost (Doroff and Keith 1990; Costanzo et al. 1995). Similarly, the low resistance exhibited by hatchling *C. picta bellii* under certain environmental conditions (Costanzo et al. 1998, 2000*a*, 2000*c*; Packard et al. 1999) likely improves its ability to tolerate somatic freezing (Storey et al. 1988; Churchill and Storey 1992; Costanzo et al. 1995; Packard et al. 1999).

*Desiccation Resistance in Hatchling Turtles*

Ectotherms that hibernate on land, particularly within the frost zone, may contend with chronic water stress (Gregory 1982; Danks 2000). Cutaneous water uptake has been reported for fossorial lizards burrowed in moist substratum (Noble and Mason 1932; Bogert and Cowles 1947) and for turtles immersed

Table 3: Least squares regressions of evaporative water loss (EWL) or resistance to inoculative freezing, as indicated by the temperature of crystallization ($T_c$) of turtles immersed in a frozen substratum, on morphological measurements of exposed skin in hatchlings of eight turtle taxa

| Dependent Variable | Independent Variable | Equation | $r^2$ | $F$ | $P$ |
|---|---|---|---|---|---|
| EWL (mg $h^{-1}$) | Critical exposure area | $y = -.20 + .004x$ | .64 | 10.5 | .018 |
| EWL (mg $g^{-1}$ $d^{-1}$) | Critical exposure index | $y = -2.39 + 3.56x$ | .48 | 5.4 | .059 |
| $T_c$ (°C) | Critical exposure area | $y = -2.50 + .001x$ | .22 | .3 | .631 |
| $T_c$ (°C) | Critical exposure index | $y = -3.82 + .84x$ | .52 | 5.4 | .069 |

Note. Coordinates for *Chrysemys picta bellii* were omitted from regressions involving inoculative freezing (df = 1, 5). Critical exposure index is the ratio of carapacial area to plastral area.

in water (Bentley and Schmidt-Neilsen 1970; Chessman 1984). However, with the possible exception of *T. ornata*, our hatchling turtles apparently were unable to reabsorb lost body water from surrounding soil, indicating that terrestrial hibernators likely remain in water deficit until after spring emergence. Our finding casts doubt on the supposition that water balance in hibernating *C. picta bellii* tracks changes in soil moisture levels (Costanzo et al. 1995) but underscores the importance of desiccation resistance as a preadaptation to terrestrial hibernation.

With the exception of *S. carinatus*, our hatchling turtles survived the loss of a modest amount of body water. Our EWL data for various taxa, whose habits range from wholly terrestrial (*T. ornata*) to highly aquatic (*S. odoratus* and *S. carinatus*), support the tenet that desiccation resistance in turtles correlates with habitat aridity (Bogert and Cowles 1947; Bentley and Schmidt-Nielsen 1966, 1970; Ernst 1968; Seidel and Reynolds 1980; Chessman 1984; Stone and Iverson 1999). The high rates of EWL exhibited by aquatic turtles may explain why hatchlings of these species commonly hibernate underwater rather than on land, even in regions where winters are mild.

The extent to which hatchling turtles dehydrate during terrestrial hibernation is unknown. Our preliminary observations in the Nebraska Sandhills showed that hatchlings excavated in April 2000 from within (*C. picta*; $n = 10$) or below (*T. ornata*;

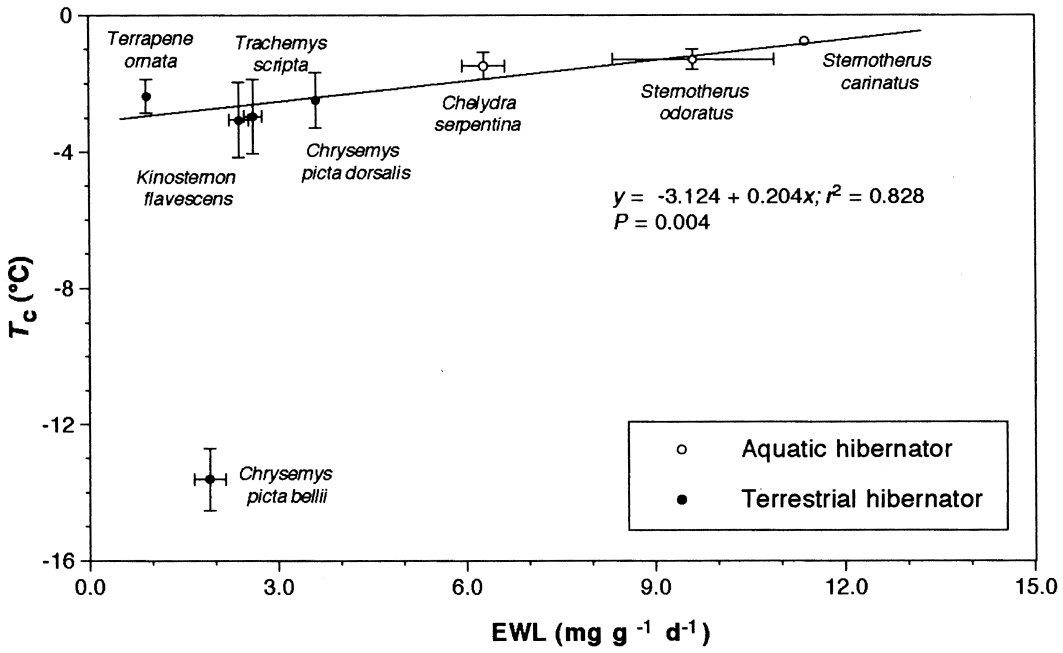


Figure 3. Relationship between susceptibility to ice inoculation, as indicated by the temperature of crystallization ($T_c$) of turtles immersed in frozen substratum ($n = 4$–9), and the rate of evaporative water loss (EWL; $n = 3$–10). Means are shown ±1 SE. Regression analysis excluded data for *Chrysemys picta bellii*.

$n = 3$) marked nests and then held overnight in shallow water at approximately 20°C gained 11.4% ± 1.1% and 6.9% ± 2.3%, respectively, of their body mass. Whether survival of these species may be compromised during especially cold and dry winters is unknown.

### *Morphological Correlates of Resistance to Desiccation and Inoculative Freezing*

Evaporation from cutaneous (as opposed to respiratory) surfaces usually accounts for the majority of EWL in reptiles (Bentley and Schmidt-Nielsen 1966; Claussen 1967; Chessman 1984). Among adult turtles, the carapacial and plastral scutes of the shell better resist water loss as compared with the softer epidermis of the head, neck, and limbs (Rose 1969; Spotila and Berman 1976). We presume that the same is true of hatchlings. Therefore, the interspecific variation in EWL we observed chiefly reflected differences in body size and shape (i.e., surface/volume relationship) and, also, shell morphology, which in part determines cutaneous surface area (Wygoda and Chmura 1990; Stone et al. 1992; Stone and Iverson 1999). EWL is also influenced by cutaneous permeability (Spotila and Berman 1976), which is likely governed by degree of keratinization (Seidel and Reynolds 1980) and/or lipid content (Roberts and Lillywhite 1980; Lillywhite and Maderson 1988) of the epidermis, but this attribute has not been extensively studied in turtles. In our hatchlings, EWL was influenced by the area of skin at the openings to the limb and nuchal pockets, which in turn was related to body size and shell morphology (Tables 2, 3). Furthermore, the weak association between EWL and a size-independent index of skin exposure (Table 3) suggests either taxonomic variation in skin permeability or simply that turtles having reduced plastrons (i.e., aquatic species) are particularly susceptible to EWL.

The mechanism(s) by which ice nuclei infiltrate the bodies of ectothermic animals is not adequately understood. Among hatchling turtles, cutaneous transmission is important (Packard and Packard 1993*a*), although ingress of ice nuclei may also occur via the orifices (e.g., cloacal, nostrils, eyes). With exclusion of *C. picta bellii*, EWL was an excellent predictor of inoculation resistance in hatchling turtles (Fig. 3). This finding not only suggests that a similar morphological attribute governs resistance to both desiccation and inoculative freezing but may also explain the greater propensity for inoculation resistance among terrestrial hibernators. For example, the more extensive plastron found in these species may limit exposure of permeable skin and may shield the mucous membranes of the cloaca from contact with environmental ice nuclei. Notably, inoculation resistance is exceptionally well developed in *C. picta bellii*, which must survive exposure to subzero temperatures while overwintering within its natal nest. The specialized adaptations promoting inoculation resistance in this species are as yet unknown but apparently do not involve blood-borne antifreezes (Costanzo et al. 2000*b*). Possibly, this attribute reflects characteristics of the integument that are unique to this species.

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