

Cold-hardiness and dehydration resistance of hatchling Blanding's turtles (*Emydoidea blandingii*): implications for overwintering in a terrestrial habitat

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Abstract: The overwintering habits of hatchling Blanding's turtles, *Emydoidea blandingii* (Holbrook, 1838), are not well understood. To ascertain whether these turtles are well suited to hibernation on land, we examined susceptibility to dehydration, supercooling capacity, resistance to inoculative freezing, capacity for freeze tolerance, and physiological responses to somatic freezing in laboratory-reared, hatchling *E. blandingii*. Rates of evaporative water loss (mean \pm SE = $4.1 \pm 0.2 \text{ mg}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$) were intermediate to rates previously reported for the hatchlings of species known to hibernate on land and in water. Supercooled hatchlings recovered from a 1-h exposure to -8°C or a 7-d exposure to -4°C . Additional turtles supercooled to $-14.3 \pm 1.2^\circ\text{C}$ (mean \pm SE) before spontaneously freezing. However, when immersed in frozen soil, their capacity to supercool was severely limited by an inability to resist inoculative freezing following contact with external ice and ice nuclei. Therefore, hatchlings likely do not use supercooling as a winter survival strategy. Hatchlings tolerated a 72-h period of somatic freezing to -3.5°C and responded to somatic freezing by increasing plasma concentrations of the putative cryoprotectants lactate and glucose. Our results suggest that hatchling *E. blandingii* could overwinter in moist, terrestrial hibernacula where risk of dehydration is reduced and freeze tolerance is promoted.

Résumé : Les habitudes hivernales des tortues mouchetées, *Emydoidea blandingii* (Holbrook, 1838), néonates sont mal connues. Nous avons examiné la sensibilité à la déshydratation, la capacité de surfusion, la résistance au gel par inoculation, la tolérance au gel et les réactions physiologiques au gel somatique chez des *E. blandingii* néonates élevés en laboratoire afin de déterminer s'ils sont bien adaptés à l'hibernation dans le sol. Les taux de perte d'eau par évaporation (moyenne \pm erreur type = $4,1 \pm 0,2 \text{ mg}\cdot\text{g}^{-1}\cdot\text{j}^{-1}$) sont intermédiaires entre ceux rapportés dans la littérature chez les tortues néonates qu'on sait hiberner dans le sol et celles qui hibernent dans l'eau. Les tortues néonates en surfusion survivent à une exposition de 1 h à -8°C et de 7 j à -4°C . D'autres tortues sont restées en surfusion à $-14,3 \pm 1,2^\circ\text{C}$ (moyenne \pm erreur type) pour ensuite geler spontanément. Cependant, une fois couvertes de sol gelé, la capacité de surfusion des tortues est considérablement réduite à cause de leur incapacité à résister au gel par inoculation au contact de glace externe et de noyaux de glace. Les tortues néonates n'utilisent sans doute pas la surfusion comme stratégie de survie à l'hiver. Les tortues tolèrent une période de 72 h de gel somatique jusqu'à $-3,5^\circ\text{C}$ et réagissent à ce gel en augmentant leur concentrations plasmatiques de substances cryoprotectrices probables, le lactate et le glucose. Nos résultats laissent croire que les *E. blandingii* peuvent passer l'hiver dans des hibernacles terrestres humides dans lesquels le risque de déshydratation est réduit et où la tolérance au gel est favorisée.

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Introduction

Freshwater turtles inhabiting northern latitudes are faced with the challenge of surviving cold winters. The hatchlings of most northern species emerge from their natal nests in autumn and overwinter in aquatic habitats where temperatures probably remain above zero, although they may encounter hypoxic or anoxic conditions that could cause osmotic and

acid-base perturbations (Ultsch 1989). The hatchlings of a few northern species overwinter in terrestrial habitats, apparently to avoid predation, and defer emergence until a time when resources are increasing (Gibbons and Nelson 1978). However, terrestrial hibernation also poses certain challenges. For example, owing to the low water potential of frozen soil, hatchlings hibernating within the frost zone may be subjected to dehydration (Costanzo et al. 2001b). In addi-

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tion, they may encounter temperatures that fall below the equilibrium freezing/melting point of their body fluids.

Generally, hatchling turtles cope with subzero temperatures either by avoiding freezing via supercooling (i.e., remaining liquid below the freezing point of their body fluids) or by tolerating the freezing of their extracellular fluids and the attendant cellular dehydration (Storey et al. 1988; Costanzo et al. 1995). Freeze tolerance and supercooling are usually mutually exclusive survival strategies because environmental conditions promoting the former also constrain the latter (Packard and Packard 1993; Costanzo et al. 1998). Nevertheless, at least some species of small reptiles may employ either mechanism, depending upon particular environmental circumstances (Lee and Costanzo 1998).

The Blanding's turtle, *Emydoidea blandingii* (Holbrook, 1838), has a northerly distribution that ranges from Nova Scotia to the Great Lakes region and west to Nebraska (Ernst et al. 1994). Adults are known to overwinter in marshes and other permanent water bodies (e.g., Kofron and Schrieber 1985; Joyal et al. 2001), but the hibernacula used by hatchlings have not been described. The literature suggests that hatchlings may overwinter in terrestrial habitats (Congdon et al. 1983, 1993, 2000; Butler and Graham 1995; Standing et al. 1997; McNeil et al. 2000; Pappas et al. 2000) or under water (Packard et al. 1999), although no study has provided conclusive evidence for either site.

The aim of this study was to ascertain whether hatchling *E. blandingii* are adapted to survive winter on land. Specifically, we examined their susceptibility to dehydration, supercooling capacity, resistance to inoculative freezing (i.e., initiation of internal freezing by contact with environmental ice or ice nuclei), capacity for freeze tolerance, and physiological responses to somatic freezing. We intended that these results would provide clues to the actual hibernation sites used by these turtles, as well as information about the comparative biology of hatchling turtles.

Materials and methods

Animals

Adult female Blanding's turtles were trapped using fyke nets or collected by hand at Beem Lake in Hyannis, Nebraska, during June 2001 and 2002. Gravid females ($n = 14$) were administered synthetic oxytocin via syringe and needle to induce oviposition (Ewert and Legler 1978). After injection, females were placed in large tubs containing warm tap water and allowed to oviposit. We grouped the eggs by clutch and incubated them at 28 °C on a substratum of vermiculite (1.0 g water/g dry vermiculite) in an environmental chamber (Model I-35X, Percival, Boone, Iowa). The resulting hatchlings weighed 6.76 ± 0.10 g (mean \pm SE; $n = 77$) and measured 31.5 ± 0.6 mm in carapace length. They were not sexed, but it is likely that both males and females were produced at this incubation temperature (Gutzke and Packard 1987).

Hatchlings were kept in darkness and cold acclimated by incrementally reducing the temperature of the environmental chamber from an initial 20 °C in September to 15 °C in October, 10 °C in November, and, finally, 4 °C in December (Costanzo et al. 1995). Food and water were withheld during this period because hatchlings probably do not eat or drink

during winter. Experiments were conducted between December and March using cold-acclimated turtles. In most experiments, a single hatchling from each clutch was used in each experiment to avoid maternal effects. Surviving hatchlings were released at the collection site in late spring. Animal care and experimental procedures were approved by the Animal Care and Use Committee of Miami University in accordance with guidelines established by the United States Public Health Service and the Canadian Council on Animal Care.

Dehydration resistance

Resistance to dehydration was gauged from the rate of evaporative water loss (EWL) for hatchlings measured under controlled laboratory conditions (Costanzo et al. 2001b). Hatchlings ($n = 6$) were held overnight (at 4 °C) in open plastic cups to permit evaporation of excess moisture from the body surfaces. Next, they were gently brushed to remove any adherent vermiculite, weighed to the nearest 0.01 mg on an electronic balance (Model AG245, Mettler-Toledo, Hightstown, New Jersey), and placed individually within screen stalls inside a dehydration chamber. The chamber was continuously ventilated with cold air (4 °C, 75% RH) at 250 mL/min, a rate that replaced the air volume approximately 2.5 times/h. Hatchlings were weighed 10 d later and EWL was calculated from the decrease in body mass, which chiefly reflected cutaneous and pulmonary water loss because the turtles did not urinate or defecate. To assess their tolerance to dehydration, we determined whether each turtle would respond to prodding with a blunt probe. Subsequently, the hatchlings were euthanized by severing the spinal cord with scissors and their carcasses were thoroughly dried (to constant mass) in a 65 °C oven for approximately 1 week. We calculated the water content of the carcasses from the mass lost upon drying.

Supercooling capacity and tolerance to extreme cold

Hatchlings ($n = 7$) were prepared for use in supercooling trials by holding them overnight (at 4 °C) in open plastic cups to permit evaporation of surface moisture and then brushing them to remove any adherent vermiculite. They were placed singly inside plastic tubes covered with a piece of plastic foam and outfitted with a 30-gauge copper-constantan thermocouple positioned near the carapace (Costanzo et al. 2000). The tubes were suspended in a programmable ethanol bath (Model RTE 140, Neslab, Portsmouth, New Hampshire) that was chilled at -0.5 °C/h until each turtle had produced a freezing exotherm (i.e., the release of latent heat of fusion during the formation of ice), as registered on a data logger (Omega RD-3572, Stamford, Connecticut). The temperature of crystallization (T_c) was taken as the lower limit of supercooling for each turtle.

Because chilling may be injurious or lethal to supercooled hatchlings (Hartley et al. 2000), we tested the tolerance of hatchling *E. blandingii* to a brief exposure to extreme cold and to prolonged exposure to moderate cold. For these trials, turtles were prepared as described above and chilled (-0.5 °C/h) to either -8 or -4 °C. Turtles in the former group ($n = 7$) were held at the target temperature for 1 h, whereas those in the latter group ($n = 7$) were held for 1 week, before being warmed slowly to room temperature.

We then assessed the survival status of each turtle by observing its response to gentle pinching and extension of its appendages.

Resistance to inoculative freezing

Capacity to resist inoculation by external ice was gauged by determining the T_c of hatchlings cooled in a matrix of frozen soil (Costanzo et al. 2000). Turtles ($n = 6$) were prepared for use in inoculative freezing trials as described above. On the day before the trial, they were placed individually in plastic tubes containing ~40 g of chilled, moist soil that was firmly tamped around the animal to ensure its complete and uniform exposure to the substratum. Next, the space above the soil was filled with plastic foam and the turtles were habituated to the soil for 24 h. We initiated the trials by immersing the tubes in a programmable refrigerated bath and, after their contents had attained thermoequilibrium at -0.4 °C (a temperature at which the soil, but not the turtles, could freeze), inoculated the substratum with small ice crystals. After allowing ~1 h for the soil moisture to freeze, the temperature inside the tube was reduced at 0.5 °C/h until each turtle produced a freezing exotherm.

The substratum used in our inoculation resistance trial was identical to that used by Costanzo et al. (1998). A composite of seven soil samples collected at a turtle nesting site was sieved with a 2-mm² mesh, mixed (9:1) with fine clay, and then autoclaved to destroy any organic ice-nucleating agents (INAs). These INAs include, but are not limited to, bacteria or parts of bacteria that will cause the formation of an ice crystal. Their presence may be beneficial to organisms that rely on freeze tolerance because INAs can cause freezing at high subzero temperatures; however, they may be detrimental to organisms that are freeze intolerant. The soil was dried in an oven at 65 °C for 48 h and then hydrated (0.075 g water/g dry soil) with autoclaved, ultrapurified water (Costanzo et al. 2000).

Freeze tolerance

In freeze tolerance trials, we determined the survival status of hatchling *E. blandingii* subjected to standardized episodes of somatic freezing. A group of 3–4 turtles were placed inside a plastic cup and immersed in moist builder's sand (0.15 g water/g dry sand). During the trial, soil temperature was measured using two thermocouples placed amongst the turtles and logged at 30-s intervals on a chart recorder. We chilled the turtles and soil to -0 °C by placing the cups in a large beaker that was submerged in a refrigerated alcohol bath (Forma Scientific, Marietta, Ohio). Next, we inoculated the substratum by introducing a small quantity of ice. After permitting the soil to freeze for ~2 d, we cooled the bath at 1 °C/d until the turtles attained the prescribed target temperature: -2.5 , -3.5 , or -5.0 °C. Owing to the high moisture content of the substratum, inoculation of the turtles, which was heralded by the appearance of freezing exotherms, occurred at temperatures near the equilibrium tissue freezing/melting point (approximately -0.6 °C), a condition promoting freezing survival (Storey and Storey 1988). The turtles were held at the target temperature for either 24 or 72 h, after which they were gradually warmed to 4 °C in a darkened environmental chamber. Upon reaching 4 °C, the hatchlings were removed from the cups and examined to de-

termine their survival status. Hatchlings were judged to have survived freezing if they withdrew their appendages into the shell in response to gentle tactile stimulation. Individuals that failed to meet this criterion were retested every 24 h for up to 3 weeks.

Physiological responses to somatic freezing

Additional turtles were used to determine the effect of somatic freezing on plasma levels of two putative cryoprotectants, glucose and lactate. Hatchlings ($n = 8$) were subjected to somatic freezing as described above. After remaining at an equilibrium temperature of -3.5 °C for 24 h, they were rewarmed to 0 °C and quickly removed from the still frozen substratum. These turtles and a group of control animals ($n = 8$) were taken directly from their holding cages at 4 °C and then euthanized by severing the spinal cord near the cranium.

Blood was collected from the severed neck vessels into heparinized microcapillary tubes and centrifuged (2000g, 5 min) to pack the erythrocytes. Isolated plasma samples were stored in cryogenic vials at -80 °C until the assays were performed. We used enzymatic procedures to measure concentrations of lactate (No. 735, Sigma Diagnostics, St. Louis, Missouri) and glucose (No. 510, Sigma).

Results

Evaporative water loss

Mean (\pm SE; $n = 6$) body mass decreased from 7.0 ± 0.3 g to 6.6 ± 0.2 g during the 10 d that turtles were kept in the dehydration chamber. The rate of mass loss, which we assume reflected only evaporation of water from cutaneous and respiratory surfaces, was 4.1 ± 0.2 mg·g initial mass⁻¹·d⁻¹. Body water content initially was $79.5 \pm 0.9\%$ (w/w) but had decreased to $78.6 \pm 0.9\%$ by the end of the experiment. All hatchlings survived the experiment.

Supercooling capacity and cold tolerance

Hatchlings cooled to a mean (\pm SE; $n = 7$) temperature of -14.3 ± 1.2 °C (range: -17.0 to -10.2 °C) before freezing. This temperature was taken as the limit of supercooling. All seven turtles exposed to -8 °C for 1 h remained unfrozen and recovered fully from the treatment (total time in supercooled state, 34 h). One of the seven turtles exposed to -0.4 °C for 7 d spontaneously froze and died, whereas the remaining animals remained unfrozen (total period of supercooling, 184 h) and survived. Surviving hatchlings were responsive to tactile stimulation immediately upon warming.

Resistance to inoculative freezing

Hatchlings chilled in a matrix of frozen soil began to freeze at a mean (\pm SE; $n = 6$) temperature of -1.3 ± 0.1 °C (range: -1.4 to -0.8 °C). The T_c recorded for these turtles gives an index of their ability to resist inoculation by external ice and INAs under the conditions of the experiment.

Freeze tolerance

Hatchlings tolerated a 24-h episode of somatic freezing to an equilibrium temperature of -2.5 or -3.5 °C, but all turtles died when exposed to -5.0 °C (Table 1). All surviving tur-

Table 1. Survival of hatchling Blanding's turtles (*Emydoidea blandingii*) subjected to somatic freezing under various experimental conditions.

| Minimum temperature (°C) | Time at minimum temperature (h) | Total time frozen (h) ^a | No. surviving/no. tested |
|--------------------------|---------------------------------|------------------------------------|--------------------------|
| -2.5 | 24 | 105±6 | 7/7 |
| -3.5 | 24 | 91±9 | 7/7 |
| -3.5 | 72 | 144±5 | 6/7 |
| -5.0 | 24 | 162±5 | 0/7 |

^aPeriod between the appearance of the freezing exotherm during cooling and rewarming to -0.6 °C, the approximate melting point of turtle tissues. Variation in this parameter reflects the fact that hatchlings began to freeze at slightly different times. Values are mean ± SE.

tles responded to tactile stimulation within 24 h of thawing; however, none of the turtles exposed to -5.0 °C met our survival criterion.

All except one of the hatchlings subjected to a 72-h episode of somatic freezing to -3.5 °C recovered fully (Table 1), although these turtles were refractory to stimulation until ~48 h after thawing. The remaining turtle showed feeble responses on the 11th day after thawing, but it never recovered fully and therefore was euthanized.

Metabolite levels in blood plasma in response to freezing

Lactate levels in turtles subjected to somatic freezing ($35.3 \pm 4.1 \mu\text{mol/mL}$; $n = 8$) were over eight times higher ($F_{[1,14]} = 55.3$, $P < 0.0001$) than those in unfrozen controls ($4.3 \pm 0.7 \mu\text{mol/mL}$; $n = 8$). Similarly, glucose levels differed markedly ($F_{[1,14]} = 381.2$, $P < 0.0001$) between the controls ($1.8 \pm 0.1 \mu\text{mol/mL}$) and the frozen hatchlings ($8.1 \pm 1.0 \mu\text{mol/mL}$).

Discussion

Hatchling turtles overwintering on land must cope with several environmental stresses that do not affect hatchlings that hibernate under water. For example, owing to the low water potential of frozen soil, and because they probably do not eat or drink, hatchlings may remain in negative water balance throughout hibernation. Terrestrially hibernating species may be adapted to resist dehydration, as they exhibit relatively low rates of EWL (Costanzo et al. 2001b). Our estimate of EWL for hatchling *E. blandingii*, $4.1 \text{ mg}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$, was intermediate to the values reported by Costanzo et al. (2001b) for hatchlings of species that hibernate on land (range: 0.9 to $3.6 \text{ mg}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$; $n = 5$) and under water (range: 6.3 to $11.4 \text{ mg}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$; $n = 3$). This finding suggests that if hatchling *E. blandingii* overwinter on land, they must be restricted to hibernacula in moist terrestrial microenvironments where water loss would be minimized. Alternatively, perhaps hatchling *E. blandingii* are especially tolerant to dehydration and therefore do not defend their body water as well as some species. In any regard, given that EWL in hatchling turtles is influenced by body size and shell morphology (Costanzo et al. 2001b), and that this species is relatively large and has an expansive shell that covers much of its skin, we were sur-

prised that hatchling *E. blandingii* were not more resistant to dehydration in our experiments.

Our finding that hatchling *E. blandingii* spontaneously froze at temperatures near -14 °C indicates that this species has a well-developed capacity to supercool. In contrast, Packard et al. (2000) found that the limit of supercooling in hatchling *E. blandingii* was -6 °C, although the animals used in that study originated from eggs that were collected from natural nests, which likely harbored INAs that may have contaminated the hatchlings (see Costanzo et al. 1998; Lee and Costanzo 1998). In our study, eggs were kept from contacting soil-borne INAs. In addition, Packard and co-workers apparently tested their turtles following a rapid exposure to cold during the autumn. Recent study suggests that gradual acclimatization to winter conditions (as in the present study) promotes gut evacuation of endogenously produced INAs, which, in hatchling *Chrysemys picta* (Schneider, 1783), is requisite to the full development of supercooling capacity (Costanzo et al. 2003).

Recent study suggests that exposure to subzero temperatures may be injurious or lethal to hatchling turtles, even if they remain unfrozen (Hartley et al. 2000). The reduced cardiac output and tissue perfusion associated with severe chilling apparently results in a functional hypoxia, which in turn leads to an increase in lactic acid concentrations and, presumably, acid-base perturbations (Hartley et al. 2000; Costanzo et al. 2001b). We found that hatchling *E. blandingii* were not adversely affected by brief exposure to -8 °C or by prolonged exposure to -4 °C, suggesting that they are adapted to cope with hypothermic stress.

Although our discussion thus far might suggest that supercooling is an important winter survival strategy in hatchling *E. blandingii*, results of our inoculation resistance experiments indicate otherwise. To exploit an intrinsic capacity to supercool, an organism must avoid being inoculated by ice or INAs within the microenvironment (Lee and Costanzo 1998). Our results indicate that hatchling *E. blandingii* have little ability to resist inoculative freezing (see also Packard et al. 2000).

Inoculation resistance in freeze-avoiding ectotherms is highly sensitive to factors that influence the abundance and distribution of ice and INAs within their immediate surroundings, as well as the degree of intimacy between these agents and the animal (Lee and Costanzo 1998). For hatchling turtles overwintering inside their natal nest, the capacity to resist inoculation strongly depends on the moisture content of the soil, as well as its texture, composition, and porosity (Costanzo et al. 1998). The substratum used in our experiments was identical to that used in previous studies conducted in our laboratory, enabling us to compare responses of *E. blandingii* to those of other species. Among the eight taxa studied by Costanzo et al. (2001b), only two species (*Sternotherus odoratus* (Latreille, 1801) and *Sternotherus carinatus* (Gray, 1855)) whose hatchlings hibernate under water resisted inoculation as poorly as *E. blandingii*. Hatchling *E. blandingii* lack a dense, unsaturated lipid layer in the basal part of the α -keratin layer of the epidermis that may promote inoculation resistance in some species, such as *C. picta* (Willard et al. 2000). In addition, given that inoculation resistance is correlated with EWL, the abundance and (or) quality of this lipid layer may partly explain inter-

specific variation in dehydration resistance among hatchling turtles (Costanzo et al. 2001b). However, both traits are also influenced by shell morphology and, ultimately, the amount of skin exposed to the environment. Therefore, one might expect to find similarity in inoculation resistance and EWL between hatchling *E. blandingii* and hatchling *Terrapene ornata* (Agassiz, 1857) because both species possess a large, hinged plastron that reduces skin exposure. However, hatchling *E. blandingii* were considerably more susceptible to inoculative freezing and dehydration.

Freeze tolerance

Previous studies (Packard et al. 1999, 2000) of freeze tolerance in hatchling *E. blandingii* suggested that turtles (indigenous to Minnesota) could survive only brief episodes of freezing at relatively high body temperatures. For example, Packard et al. (2000) found that only 20% of their turtles recovered from a 24-h period of freezing at -2.6°C , and no turtle survived a 36-h exposure to -3.6°C . In contrast, all of our turtles tolerated a 72-h period of somatic freezing at -3.5°C . This discrepancy might reflect interpopulational variation in freeze tolerance capacity but more likely stems from differences in acclimation of the animals and the testing protocol. Packard et al. (2000) acclimated their hatchlings to cold over a period of a few weeks and apparently tested them in the autumn, whereas our turtles were gradually exposed to cold over a 5-month period and tested during winter. Acclimatization to winter conditions is critical to the development of freeze tolerance in insects (Baust and Nishino 1991), frogs (Layne and Lee 1989), and hatchling turtles (Costanzo et al. 2004). Furthermore, by assessing survival shortly after thawing, Packard et al. (1999, 2000) may have underestimated the survival rate. Results of the present study indicate that turtles may require a longer recovery period before they exhibit signs of viability.

Hatchling *E. blandingii* appear well adapted to use freeze tolerance as a winter survival strategy. For example, their poor resistance to inoculative freezing may serve to reduce the potential for cryoinjury associated with a rapid and uncontrolled freezing of deeply supercooled tissues (Storey and Storey 1988; Claussen and Costanzo 1990). Similarly, hatchlings of the closely related *T. ornata* are freeze tolerant (Costanzo et al. 1995) and also are highly susceptible to inoculative freezing (Costanzo et al. 2001b). Furthermore, hatchling *E. blandingii* apparently tolerate tissue ischemia resulting from arrest of the oxygen delivery system during freezing. Our turtles accumulated large amounts of lactate and glucose during freezing, but whether these compounds play a cryoprotective role, or were elevated simply as a consequence of a generalized response to hypothermia, freezing, or hypoxic ischemia, is unknown. Similar patterns of hyperlactemia and hyperglycemia have been observed in other hatchling turtles during freezing, supercooling, or anoxia (Storey et al. 1988; Churchill and Storey 1992; Costanzo et al. 2001a).

Our results suggest that *E. blandingii* belongs to a small group of turtles that have developed a profound capacity to tolerate somatic freezing. This group includes hatchling *C. picta* (Storey et al. 1988), *T. ornata* (Costanzo et al. 1995), *Terrapene carolina* (L., 1758) (Costanzo and Claussen 1990), and *Trachemys scripta* (Schoepff, 1792) (Churchill

and Storey 1992). All of these species belong to the Emydidae, a large family of mostly North American pond turtles that includes *E. blandingii*, and hatchlings of all are known to hibernate terrestrially, even at the northern limit of their geographic distribution. In contrast, experiments on taxa within the Kinosternidae, Trionychidae, and Chelydridae showed that only hatchling *Chelydra serpentina* (L., 1758) (Chelydridae) tolerated even a minimal amount of freezing (Costanzo et al. 1995; Packard et al. 1999). Hatchlings of these species typically hibernate under water or deep in the soil, where they are protected against frost exposure. Contrary to the conclusions of Packard et al. (1999), freeze tolerance is not a common attribute among hatchling turtles. We suspect that the ability of *E. blandingii* to survive freezing may be relevant to its winter life history.

Clues to the overwintering sites of hatchling *E. blandingii*

Although the hibernation habits of hatchling *E. blandingii* are not known with certainty, authors of several studies (Congdon et al. 1993, 2000; Butler and Graham 1995; Standing et al. 1997; McNeil et al. 2000; Pappas et al. 2000) suggested that overwintering on land is at least possible, if not probable. Some of these studies showed that hatchlings emerge from their nests in autumn, although they may ultimately occupy winter refugia elsewhere on land, rather than under water. In fact, Standing et al. (1997) and McNeil et al. (2000) found that most hatchlings did not seek water after emergence from the nest and actually avoided water when released next to it. Furthermore, although several hatchlings initially entered water, they soon emerged and moved toward vegetated terrestrial habitats. It is possible that hatchlings emerge from the nest in the fall and seek terrestrial overwintering sites that may be more suitable. The nest chamber may be inhospitable because of low water potential (i.e., risk of dehydration) and severe temperatures due to its shallow depth. Hatchlings may ultimately seek moist and friable soils where they can burrow, maintain water balance, and avoid severe cold. Possible microhabitats include burrows beneath vegetation or refugia near wetland edges or within the soil column below the nest chamber. In contrast, on the basis of their laboratory results, Packard et al. (2000) concluded that hatchling *E. blandingii* must overwinter under water or else risk death due to freezing, although they offered no corroborating evidence from field studies.

Clearly, carefully conducted field studies are needed before the winter habits of this species will be elucidated. Results of the present study suggest that hatchling *E. blandingii* could successfully overwinter in the nest or elsewhere on land, provided that their immediate surroundings remain moist (to both retard dehydration and promote inoculative freezing) and relatively warm (above -3.5°C). Studies of the microenvironmental conditions within the hibernaculum of other hatchling turtles (e.g., Costanzo et al. 1995; Nagle et al. 2000) suggest that these conditions are routinely met throughout much of the geographic range of *E. blandingii*. However, in locales where the climate is more extreme, such as the Nebraska Sandhills, much colder and drier winters commonly occur (see Costanzo et al. 2004). In these areas, hatchling *E. blandingii* may avoid lethal cold by burrowing deeply into the sandy, friable soil (McNeil et al. 2000). This

appears to be the strategy used by the sympatric *T. ornata* and *Kinosternon flavescens* (Agassiz, 1857) (Costanzo et al. 1995). Field studies that identify definitive overwintering sites, as well as the environmental stresses to which they are exposed, would contribute greatly to our knowledge of the winter life history of this species.

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