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Adaptations to terrestrial overwintering of hatchling northern map turtles, *Graptemys geographica*

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Abstract We conducted a 3-year field and laboratory study of winter biology in hatchlings of the northern map turtle (*Graptemys geographica*). At our study area in northern Indiana, hatchlings routinely overwintered in their natal nests, emerging after the weather warmed in spring. Winter survival was excellent despite the fact that hatchlings were exposed frequently to subfreezing temperatures (to −5.4 °C). In the laboratory, cold-acclimated hatchlings exhibited low rates of evaporative water loss (mean = 2.0 mg g^{-1} day^{-1}), which would enable them to conserve body water during winter. Laboratory-reared hatchlings were intolerant of freezing at −2.5 °C for 24 h, conditions that are readily survived by freeze-tolerant species of turtles. Winter survival of hatchling *G. geographica* probably depended on their extensive capacity for supercooling (to −14.8 °C) and their well-developed resistance to inoculative freezing, which may occur when hatchlings contact ice and ice-nucleating agents present in nesting soil. Supercooled hatchlings survived a brief exposure to −8 °C. Others, held at −6 °C for 5 days, maintained ATP concentrations at control levels, although they did accumulate lactate and glucose, probably in response to tissue hypoxia. Therefore, anoxia tolerance, as evidenced by the viability of hatchlings exposed to N_2 gas for 8 days, may promote survival during exposure to subfreezing temperatures.

Keywords Dehydration · Freeze tolerance · Supercooling · Anoxia · Inoculative freezing

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Abbreviations *EWL*: evaporative water loss · FP_{eq}: equilibrium freezing point · *INA*: ice-nucleating agents · T_c: temperature of crystallization

Introduction

Whereas the adults of most species of aquatic turtles hibernate in the unfrozen depths of ponds and rivers, their offspring commonly spend their first winter of life on land (Gibbons and Nelson 1978; Ultsch 1989). Overwintering within the natal nest may benefit the hatchlings by reducing their exposure to predators at a time when resources necessary for rapid growth are in decline (Wilbur 1975). However, at high latitudes the hatchlings of many aquatic species emerge from their nests in autumn and, like the adults, hibernate in thermally buffered aquatic habitats apparently because they are ill equipped to survive harsh winter conditions (Obbard and Brooks 1981; Packard et al. 1993; Costanzo et al. 1995, 2000b, 2001b; Packard et al. 2000; Sims et al. 2001). The hatchlings of a few northern species nevertheless hibernate terrestrially and therefore must contend with dehydrating and subfreezing conditions. Their survival during winter requires adaptations permitting them to limit water loss and tolerate exposure to subfreezing temperatures (Costanzo et al. 2001b).

Despite their northern distribution, little is known about the winter biology of hatchlings of the northern map turtle, *Graptemys geographica*. Anecdotal reports suggest that overwintering within the nest occurs in populations throughout Midwestern North America (Illinois: Cahn 1937; Indiana: Newman 1906; Costanzo et al. 2001c; Minnesota: Breckenridge 1944; Pappas et al. 2000; Wisconsin: Vogt 1981). However, no study has provided unequivocal evidence that hatchling *G. geographica* routinely overwinter within the natal nest.

If hatchling *G. geographica* do hibernate terrestrially they must cope with dehydration because the low water potential of the frozen soil matrix promotes evaporative

water loss (EWL; see Costanzo et al. 2001b). Furthermore, hatchling turtles are particularly prone to EWL because their surface area is large relative to their body mass (Mautz 1982; Nagy et al. 1997).

Hatchling *G. geographica* overwintering inside the nest may be challenged by temperatures that fall below the equilibrium freezing point (FP_{eq}) of their body fluids, ca. −0.6 °C. In this circumstance, survival may depend on a tolerance to the freezing of body fluids, or an avoidance of freezing, via supercooling, or a combination of these strategies (Storey and Storey 1996; Lee and Costanzo 1998). Both mechanisms have limitations. For example, hatchlings of freeze-tolerant species survive somatic freezing only so long as their body temperature remains above ca. −4 °C (Storey et al. 1988; Costanzo et al. 1995; Packard et al. 1999). On the other hand, because supercooled turtles are in a metastable state, ice nucleation can occur spontaneously or may be triggered by contact with ice and ice-nucleating agents (INA) in the nest microenvironment (Packard et al. 1993, 2000; Costanzo et al. 2000a). Susceptibility to inoculative freezing is strongly influenced by several characteristics of the surrounding soil, including its moisture level, texture, porosity, and organic content (Costanzo et al. 1998). Therefore, supercooling is a viable strategy only if turtles have a well-developed resistance to inoculation by external INA and lack endogenous INA (Lee and Costanzo 1998; Costanzo et al. 2003). Furthermore, because tissue perfusion is greatly reduced at such low temperatures, supercooled turtles must tolerate the physiological perturbations associated with hypoxia (Packard and Packard 1999; Hartley et al. 2000; Costanzo et al. 2001a).

The purpose of this study was to provide conclusive evidence of successful terrestrial hibernation in hatchling *G. geographica* and to characterize the micro-environmental conditions to which these hatchlings are exposed during winter. To better understand how these animals survive within the nest, we determined their capacities for dehydration resistance, freeze tolerance, supercooling, and inoculation resistance. In addition, we examined their physiological responses to supercooling and anoxia by measuring the concentrations of key metabolites for individuals exposed to subfreezing and anoxic conditions.

Materials and methods

Study area and field observations

During 1999–2002 we studied a population of *G. geographica* inhabiting Mount Zion Millpond, Fulton County, Ind. (41°N, 86°W), in the northern portion of this species' geographic range. Winters in this region are generally cold and wet (>20 cm rain), although frost penetration into the soil may be limited by the insulating effect of heavy snow cover that often persists for long periods each winter.

We followed females during their nesting forays and protected their nests from predators by securing a raised, protective screen above the nest. The nests were monitored daily from September to July to determine the dates of hatchling emergence. Clutch size was determined by excavating the nests after emergence ceased in early July and totaling the number of failed eggs, dead hatchlings, and hatchlings that had emerged. Depth to the top egg and maximum nest chamber depth were recorded for each nest.

Winter microenvironment

We deployed a miniature temperature logger (HOBO Stowaway XTI and/or Tidbit, Onset Computer, Pocasset, Mass.) within the soil column adjacent to the chambers of several nests. At four permanent stations located at the study area, we used additional loggers to record the temperature at depths (5, 10, and 15 cm) bracketing the zone in which most turtles nest. These stations were widely separated and broadly representative of the range of thermal environments available to nesting turtles. The resulting data were analyzed to determine winter minimum temperature, the number of chilling episodes in which temperature fell below the FP_{eq} of body fluids ("critical chilling episodes"), and the total time that temperature remained below this benchmark ("critical exposure time").

We measured the soil moisture level at the study site periodically during winter 2001–2002. Soil samples, collected on 17 November, 24 December, 24 January, and 25 February using an auger, were taken at a depth of 10 cm at each of the four permanent stations as well as adjacent to the chamber of three turtle nests (nos. 01–4, 01–19, and 01–54). Moisture content of the samples was determined from the change in mass during drying in a 65-°C oven and expressed as a percentage of the dry soil mass.

Acclimation and use of hatchlings in laboratory experiments

For our laboratory experiments, we trapped gravid turtles from a nearby (<2 km) pond and induced oviposition with synthetic oxytocin (Ewert and Legler 1978). Eggs collected in this manner were placed on moistened vermiculite (1.0 g water g^{-1} vermiculite; approximately −150 kPa) in clean containers and were prevented from making contact with soil or water, which may harbor INA. In the laboratory, we grouped the eggs by clutch and incubated them at 28 °C in an environmental chamber (model I-35X, Percival, Boone, Iowa) until they hatched. Moisture content of the substratum was adjusted periodically by weighing the containers and replacing any water lost by evaporation.

Hatchlings were gradually acclimated to 5 °C, in darkness, following a protocol intended to represent conditions in natural nests (Costanzo et al. 2000b). After hatching, turtles were held at 22 °C until 1 September, when the temperature was lowered to 20 °C. Further decrements in ambient temperature were instituted on 1 November (10 °C) and on 1 December (5 °C) and the hatchlings were thereafter held at 5 °C until used in experiments.

We maximized the number of clutches represented in each experiment in order to avoid the potentially confounding influence of genetic dependence. In some cases siblings were present in the same experiment, though never within the same treatment group. Turtles weighed 5.41 ± 0.08 g and were 29.4 ± 0.1 mm in carapace length (mean ± SEM; $n = 106$) at hatching.

Dehydration susceptibility

Resistance to dehydration was inferred from the rate of EWL from hatchlings exposed to an atmosphere of low water potential. Each hatchling ($n = 7$) was brushed to remove adhering vermiculite, air-dried to remove surface moisture, and weighed to the nearest 0.1 mg on a balance (Mettler-Toledo, model AG245 Hightstown, N.J.). The turtles were placed individually in stalls (6×8 cm) inside a dehydration chamber that was held at 5 °C and continuously ventilated with cold air (5 °C, 75% RH) at a flow rate (250 ml min^{-1}) which was sufficient to replace the air volume 2.5 times h^{-1} (Costanzo et al. 2001b). We reweighed the turtles after 24, 144 and

240 h and calculated EWL (mg water g^{-1} day^{-1}) from the decrease in body mass. We assumed that the decrease in body mass reflected only evaporative loss via cutaneous and respiratory routes because the turtles did not urinate or defecate.

Freeze tolerance

We used the protocol of Costanzo et al. (1995) to evaluate tolerance of somatic freezing in hatchling *G. geographica*. Hatchlings (n=4–5) were placed in a shallow plastic container and covered with damp sand (0.15 g water g^{-1}; water potential = −105 kPa). The measuring junctions of two copper-constantan thermocouples were positioned in the sand, adjacent to the cluster of hatchlings, in order to record the temperature (30-s intervals) on a data logger (Omega OM 500, Stamford, Conn.). The containers were allowed to equilibrate to −0.4 °C in a refrigerated bath (Forma 2095, Forma Scientific, Marietta, Ohio). Next, we inoculated the substratum with ice crystals and permitted the water in the matrix to freeze completely (ca. 24 h) before cooling the turtles at approximately 0.1 °C h^{-1} to the prescribed target temperature. The onset of freezing of the turtles, evidenced by the appearance of a freezing exotherm, occurred at ca. −1 °C. We held the turtles (n=5–10 per treatment group) at the prescribed target temperature, −2.5 °C or −3.5 °C, for either 24 h or 48 h.

Hatchlings were thawed by slowly raising the bath temperature to 5 °C. We then determined the survival status of the turtles by monitoring their response to tactile stimulation and capacity for locomotion during the ensuing 7-day period. Turtles that were incapable of locomotion or otherwise impaired were killed and included in mortality statistics for the experiment.

Supercooling capacity and survival at subzero temperature

We determined the supercooling capacity of hatchling *G. geographica* by progressively cooling turtles until they spontaneously froze (Costanzo et al. 1995). On the day before testing, hatchlings were removed from their holding boxes, gently brushed to remove any adhering vermiculite, and held overnight in a clean, dry plastic container. This procedure eliminated surface debris and moisture that might seed the freezing of turtle tissues. We then placed each hatchling upright inside a clean plastic tube (33×105 mm) and filled the space above it with insulative plastic foam. The tubes were immersed in a refrigerated ethanol bath (Model RTE 140 Neslab; Portsmouth, N.H.), equilibrated at 0 °C, and then cooled at 0.5 °C h^{-1} until the turtles spontaneously froze. A 30-gauge copper-constantan thermocouple placed in contact with the plastron was used to record temperature on a data logger (Omega RD-3572, Stamford, Conn.). The temperature of crystallization (T_c), the lowest temperature attained before freezing, was taken as the limit of supercooling.

A companion experiment was conducted to determine whether hatchlings could survive exposure to extreme cold in the supercooled state. Hatchlings were prepared for experimentation and slowly chilled (0.5 °C h^{-1}) as described above, except that they were prevented from cooling below −8 °C. After remaining at this temperature for 1 h they were warmed at 0.5 °C h^{-1} to 5 °C (total period of supercooling = 33 h) and then examined to assess their condition using the same criteria as employed in the freeze tolerance experiments.

Physiological responses to supercooling and anoxia

Additional hatchlings (n=5) prepared as per the supercooling experiments were cooled by 2 °C each day until they attained an ultimate temperature of −6 °C. After remaining at this temperature for 5 days, they were removed from the refrigerated bath and immediately frozen by plunging them into liquid N_2. We prepared whole-body homogenates following the methods of Costanzo et al. (2001a) and assayed the supernatant for concentrations of ATP (Sigma, St. Louis, Mo., USA; no. 366), glucose (Sigma, no. 510), and lactate (Sigma, no. 735). Metabolite concentrations were expressed as micromoles per gram fresh body mass and were compared to values for control animals (n=5), which were taken directly from their holding boxes at 5 °C.

For comparison, we measured the same metabolites in anoxia-exposed hatchlings (n=5) that were placed individually on a foam platform resting inside an inverted 500-ml flask, which we then flushed copiously with gaseous N_2 and sealed with a rubber stopper. The turtles remained inside the flasks, in darkness and at 5 °C, for 8 days. We then determined their viability status, removed them from the flasks, and immediately froze them in liquid N_2. Their carcasses were assayed for ATP, glucose, and lactate as described above.

Inoculation resistance

Turtles were cleaned and prepared for testing as per the supercooling trials. They were placed upright inside a clean plastic tube (33×105 mm), instrumented with a thermocouple probe placed against the plastron, and then immersed in a volume of prepared soil (see below). A soil:turtle body mass ratio of approximately 4:1 was used in the experiments. This preparation provides enough soil to completely envelope the turtle but not so much as to obscure the turtle's exotherm (see Costanzo et al. 1998). A piece of porous plastic foam was used to insulate the space above the soil, also aiding detection of the turtle's exotherm. Turtles were habituated within the tubes for 24 h (5 °C, in darkness) before the inoculation trials commenced.

To initiate the trial, tubes were placed vertically into a refrigerated ethanol bath and chilled to ca. −0.5 °C, a temperature at which the soil, but not the turtle, could freeze. We inoculated the soil with several small ice crystals and permitted it to thoroughly freeze over the next 3–4 h. Subsequently, the temperature inside the tube was reduced at 0.5 °C h^{-1} until we observed an exotherm, indicating the crystallization of turtle tissues. The resulting T_c reflected the ability of the turtle to resist inoculative freezing under the conditions of the trial.

The soil used in these trials was a composite of samples collected within the soil column adjacent to *G. geographica* nests during mid-winter. The material was oven-dried for 48 h at 65 °C and then re-hydrated with autoclaved, deionized water to one of five levels (0, 4, 8, 12, or 16% w/w), which were chosen to bracket the range of soil moisture levels occurring within natural nests. This method of preparation presumably does not alter the activity of the constituent INA (Costanzo et al. 2000a); therefore, in these trials turtles were exposed to ice as well as native INA. We tested five turtles in each moisture treatment.

Statistical analyses

Means are reported ± SEM unless otherwise indicated. Student's t-tests were used to compare metabolite concentrations of experimentally treated turtles with those of control animals. Mean T_c values were compared among treatment groups in the inoculation resistance trials using analysis of variance (ANOVA), followed by Tukey's multiple contrasts. Statistical significance was set at $P \leq 0.05$.

Results

Terrestrial hibernation of hatchling *G. geographica*

We monitored the fate of 24 *G. geographica* nests during the three winters of our study. Three of these

nests contained only unhatched eggs and one, devoid of eggs or hatchlings, apparently had been depredated; however, 19 of the remaining 20 nests produced hatchlings that emerged in spring (Table 1). Winter mortality during the study was limited to a single hatchling found in a nest that also contained nine live hatchlings.

Three hatchlings that had emerged from one nest in late summer 1999 were found dead beneath the screen cover. We suspect that these hatchlings succumbed to dehydration and that the early emergence was an anomaly triggered by an extended period of summer drought. Upon excavation of this nest we found five additional hatchlings that were alive, albeit severely dehydrated.

Characteristics of the winter environment

Hatchling *G. geographica* overwintered in nests only ca. 12 cm below the ground surface (Table 1). Consequently, they experienced numerous episodes of chilling to subfreezing temperatures and remained at risk of freezing for extended periods (Table 1). Seasonal minimum temperatures inside these nests were as low as −5.4 °C. Relative to the nests, frost did not penetrate as deeply at the four permanent soil stations. For example, the minimum temperature recorded at the 10-cm stratum was only −0.2 ± 0.4 °C (range: −0.8 °C to 0.2 °C) in winter 2000–2001, and only −1.6 ± 1.6 °C (range: −3.3 °C to −0.1 °C) in winter 2001–02.

Moisture content of soil in the vicinity of three nests averaged ca. 8–9% (overall range: 5.0–12.6%) and did not vary appreciably during winter. Moisture levels in the soil at the four permanent stations tended to be higher, averaging 12–15% (overall range: 5.6–23.8%) during the winter.

Dehydration susceptibility trials

All hatchlings survived the 10-day exposure to dry air. The hatchlings lost 11.4 ± 0.6 mg during the first 24 h (0.48 mg h^{-1}), another 51.0 ± 3.2 mg during the next 120 h (0.43 mg h^{-1}), and an additional 39.1 ± 2.6 mg during the final, 96-h interval (0.41 mg h^{-1}). Because the rate of mass loss at the outset of the experiment was uncharacteristically high, we based our calculation of EWL on the change in body mass between the 24-h weighing and the final weighing. During this period, the turtles lost 90.2 ± 5.8 mg, or 1.8 ± 0.1% of their initial body mass, and the calculated rate of EWL was 2.0 ± 0.1 mg g^{-1} day^{-1} ($n = 7$).

Table 1 Environmental conditions associated with hibernation of hatchling *Graptemys geographica* inside the natal nest during three successive winters in northern Indiana. All hatchlings survived winter and emerged from the nest, except as indicated. *Emergence date* is the earliest date on which at least one turtle emerged from the nest

No.	Emergence date	No. hatchlings emerging	Nest chamber depth (cm)	No. critical chilling episodes	Critical exposure time (h)	Minimum nest temperature (°C)
1999–2000						
99–7	18 May	8	15	-	-	-
99–12*	9 Sep	3	-	-	-	-
99–21	28 Apr	5	-	-	-	-
99–30	8 May	6	-	-	-	-
99–46	12 May	7	9	-	-	-
99–48	24 Apr	8	-	-	-	-
99–51	9 May	8	-	-	-	-
99–59	17 May	8	-	-	-	-
Average	8 May	6.6	12	-	-	-
2000–2001						
00–12	9 Apr	13	11	19	208	−3.5
00–16	10 Apr	9	11	6	56	−2.3
00–21	4 May	9	12	11	99	−3.2
00–37	12 Apr	6	14	11	297	−1.7
00–48	3 Jun	3	12	10	99	−2.4
00–60	6 May	8	13	4	41	−1.4
Average	27 Apr	8.0	12	10	133.3	−2.4
2001–2002						
01–4	20 Apr	7	9	10	497	−5.1
01–19	25 May	6	14	9	293	−5.4
01–49	20 Apr	8	14	-	-	-
01–54	16 May	4	12	10	221	−3.6
01–82	9 May	10	10	-	-	-
01–83	26 May	9**	12	-	-	-
Average	9 May	7.3	12	10	337.0	−4.7

*These turtles emerged in autumn, possibly because they were under dehydration stress; therefore, emergence date for this record was omitted from the calculation of the group average

**One additional turtle died during winter

Tolerance of somatic freezing

Hatchlings fared poorly in our freeze tolerance trials. High mortality (9 of 10) resulted for turtles that were held at −3.5 °C for 24 h. The rapid (within 48 h) and complete recovery exhibited by the surviving turtle suggests that it had somehow avoided freezing (despite being surrounded by ice-laden soil) and remained supercooled for the duration of the experiment. However, this supposition cannot be confirmed because we did not record exotherms for individual turtles. None of the hatchlings ($n=5$) held frozen at −2.5 °C for 48 h survived. Four of the eight hatchlings held at −2.5 °C for 24 h exhibited a weak response to tactile stimulation at the end of the 7-day recovery period; however, they were unable to locomote and thus were killed.

Capacity and tolerance for supercooling

Hatchlings chilled in the absence of environmental ice and INA supercooled extensively, freezing at a body temperature of −14.8 ± 0.4 °C ($n=10$). All seven of the hatchlings exposed to a minimum temperature of −8 °C during a 33-h period of supercooling recovered fully. All were responsive to tactile stimulation within 12 h of rewarming. Most (~60%) demonstrated a capacity for locomotion within 48 h and all did so by the end of the 7-day recovery period.

Physiological responses to supercooling and anoxia

Turtles that were held at −6 °C for 5 days (8 days total exposure) in the supercooled state had whole-body ATP concentrations similar ($t=1.90$, $df=8$, $P=0.095$) to those of controls (Fig. 1). However, these turtles had comparatively higher levels of glucose ($t=10.17$, $df=8$, $P<0.0001$) and lactate ($t=6.44$, $df=8$, $P<0.0002$). We did not determine the viability status of these animals at the time of sampling; however, we presume that they were alive on the basis of their ATP concentrations.

Turtles survived the 8-day anoxia exposure without apparent ill effects. Concentrations of ATP in these turtles did not differ ($t=0.23$, $df=8$, $P=0.83$) from those in control animals (Fig. 1). Anoxic turtles had nominally higher levels of metabolites, although the comparisons with the control animals were not quite significant for either glucose ($t=1.71$, $df=8$, $P=0.13$) or lactate ($t=2.05$, $df=8$, $P=0.075$). In the case of lactate, high variability in the data (Fig. 1) probably contributed to our failure to find a significant difference.

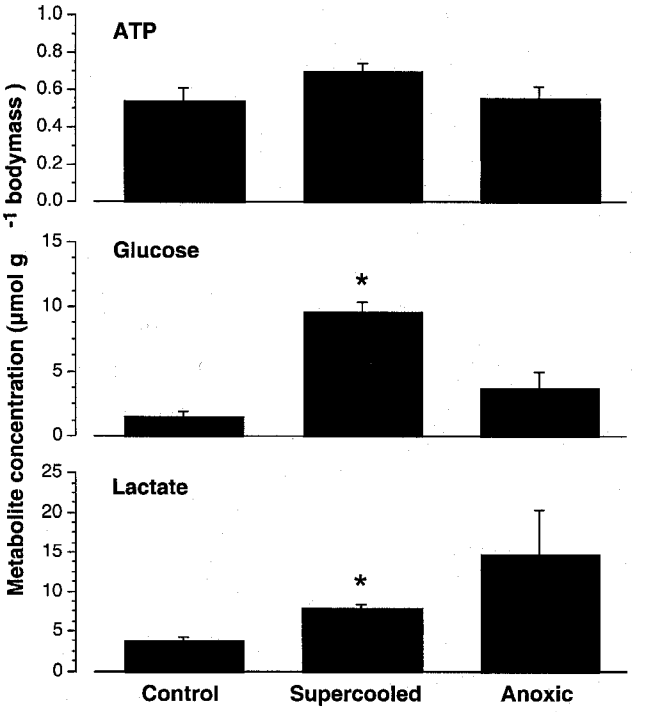

Fig. 1 Whole-body concentrations of metabolites in hatchling *Graptemys geographica* held supercooled at −6 °C for 5 days, or exposed to anoxia at 4 °C for 8 days, relative to controls. Mean values (± 1 SEM) are based on $n=5$ turtles per group. *Asterisks* denote values that differed significantly (Student's *t*-test; $P<0.05$) from the control

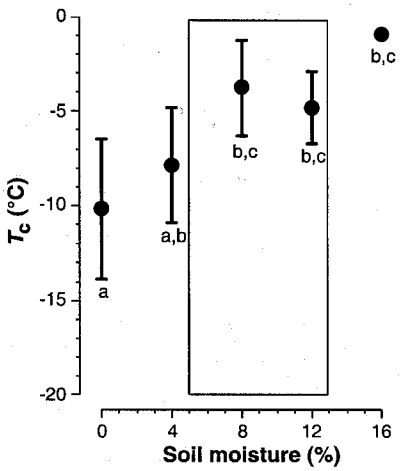

Fig. 2 Influence of soil moisture on resistance to inoculative freezing as indicated by the temperature of crystallization (T_c) of hatchling *G. geographica* immersed in a frozen substratum hydrated to various levels. Mean values (±SEM) are based on $n=5$ turtles per group. Values identified by *similar letters* were statistically indistinguishable (ANOVA, Tukey's multiple contrasts; $P>0.05$). Range of moisture levels determined for field samples of nesting soil is indicated by the *box*

Resistance to inoculative freezing

Inoculation resistance, as inferred from the T_c of turtles chilled in a pre-frozen soil matrix, was strongly influenced ($F_{4,20}=9.74$, $P<0.003$) by soil moisture (Fig. 2). Hatchlings were highly susceptible to inoculative freezing when exposed to soil with the highest moisture content (16%). In drier soil, however, they supercooled more extensively. When tested in oven-dried soil (0%),

turtles froze at -10.2 ± 3.7 °C, nearly 5 °C higher than the T_c determined for turtles in our supercooling trials.

Discussion

Observations at our study area in northern Indiana, near the northern limit of the species' range, conclusively showed that hatchling *G. geographica* regularly hibernate within the nest chamber and survive to emerge the following spring. This finding corroborates Newman's (1906) seminal report of spring emergence of hatchling *G. geographica* near Lake Maxinkuckee, Ind., ca. 35 km northwest of our study area. We found that temperatures within the nest chamber fell below freezing for extended periods during winter and attained minima as low as −5.4 °C. The limited winter mortality we observed suggests that *G. geographica* is well suited for terrestrial hibernation, even in regions where frost regularly penetrates into the soil column to the depth of their nests.

Site selection by nesting females may be an important determinant for the winter survival of hatchling turtles (Tucker and Paukstis 1999). Previous studies suggest that female *Graptemys* scrupulously avoid areas of wet sand during nesting (Cahn 1937; Vogt 1980). At our study site, *G. geographica* tended to nest in close proximity to one another and many returned to within a few meters of the same location each year. The area of highest nest density was located at the crest of a hill, adjacent to a paved road. Most of the nests were constructed in coarse loamy sand and fine gravel that probably originated as roadbed fill, although various other soils (e.g., clay, loamy clay, and sandy clay loam) occur at the study area (Costanzo et al. 2001c). An unobstructed southwestern exposure ensures that *G. geographica* nests remain in full sunlight most of the day, but our thermal and hydric data suggest that these nests are generally colder and drier during winter than other available nesting sites. Aspect, slope, and soil texture probably account for these differences.

Dehydration susceptibility

Because hatchlings overwintering within the nest chamber may be confronted with chronically dry conditions, morphological and physiological mechanisms of water conservation may be important to their winter survival. Water loss may be curbed by low ambient temperatures and a reduction in metabolic rate. Whereas overwintering hatchlings probably do not drink and are unable to absorb moisture from their surroundings (Costanzo et al. 2001b), dehydration-avoiding species may offset EWL (and perhaps even gain water) through oxidative metabolism of lipids (Gregory 1982; Wilson et al. 2001), although the rate of production of this water would be reduced at low winter temperatures. Our results indicate that hatchling *G. geographica* are well suited for terrestrial hibernation inasmuch as they are highly resistant to EWL. Indeed, among the eight taxa of turtles studied by Costanzo et al. (2001b), only the highly terrestrial *Terrapene ornata* had a perceptibly lower rate of EWL.

Freeze tolerance

In common usage, the term "freeze tolerance" refers to an ability to survive the crystallization and subsequent thawing of body fluids under naturalistic thermal and temporal regimes. Few ectotherms use freeze tolerance as a survival strategy (Storey and Storey 1996), although some species, including turtles, have a well-developed capacity to tolerate somatic freezing. For example, hatchling *Chrysemys picta* can survive freezing bouts during which body temperature falls to −4 °C and more than 50% of the body water crystallizes (Storey et al. 1988), and can recover after remaining frozen for at least 11 days (Churchill and Storey 1992a). Other species, such as hatchling *Trachemys scripta*, can tolerate a measure of freezing but their capacity seems limited to relatively high temperatures and short exposures (e.g., <24 h at −2.5 °C; Churchill and Storey 1992b). Unfortunately, detailed information regarding the winter microenvironment is lacking for many species, so it is often difficult to determine to what extent, if at all, the measured capacity promotes survival. Results of laboratory tests suggest that freeze tolerance occurs in hatchling *Chrysemys picta* (Storey et al. 1988), *Emydoidea blandingii* (Packard et al. 1999), *Terrapene ornata* (Costanzo et al. 1995), and *Trachemys scripta* (Churchill and Storey 1992b), as well as adult *Chrysemys picta* (Claussen and Kim 1993), *Terrapene ornata* (Costanzo et al. 1995) and *Terrapene carolina* (Costanzo and Claussen 1990). All of these species belong to the Emydidae, a family of mostly North American pond turtles, which includes *G. geographica*. Although freeze-tolerance trials have been conducted with taxa from the Kinosternidae, Trionychidae, and Chelydridae, only hatchling *Chelydra serpentina* (Chelydridae) tolerated even a minimal amount of freezing (Packard et al. 1993; Costanzo et al. 1995). Ultsch (1989) hypothesized that a propensity for freeze tolerance may be common among members of the Emydidae. However, our hatchling *G. geographica* did not survive freezing exposures that we deem ecologically relevant. Therefore, despite their close phylogenetic proximity (Bickham et al. 1996), *G. geographica* apparently lacks the capacity for freeze tolerance found in some other emydids. This finding is at odds with the tenuous claim that freeze tolerance is an attribute shared by the hatchlings of all species of turtles, including *G. geographica* (Packard et al. 1999; Packard and Packard 2001).

We do not know the proximate cause of death in the turtles used in our freeze tolerance trials. However, osmotic/ionic imbalance, ischemic anoxia, and metabolite end-product accumulation are all potentially lethal to freeze-intolerant animals (Storey et al. 1988). The rapid

accumulation of lactate in frozen hatchlings suggests that freezing induces an acute anoxic stress (Churchill and Storey 1992a, 1992b) and, therefore, an underlying anoxia tolerance may be requisite to freeze tolerance. Anoxia tolerance is not especially well developed in adult *G. geographica*, at least in comparison to *Chrysemys picta* (Ultsch and Jackson 1995, Reese et al. 2001). However, our tests showed that hatchling *G. geographica* can survive in a N_2 atmosphere at least 8 days, suggesting that oxygen lack is not sufficient to explain the mortality in our freeze-tolerance trials. Further study is needed to elucidate the relationship between anoxia tolerance and freezing survival in freeze-tolerant animals.

Supercooling in hatchling *G. geographica*

Lacking freeze tolerance, hatchling *G. geographica* must survive exposure to subfreezing temperatures within the nest by remaining supercooled. Our tests showed that these turtles have an exceptionally well-developed capacity for supercooling on the order of that exhibited by *Chrysemys picta* (Lee and Costanzo 1998). To achieve this level of freeze avoidance, hatchling *G. geographica* must purge any endogenous or ingested INA that would otherwise nucleate the body fluids and inhibit supercooling. This process may occur during acclimatization to winter conditions, as it does in hatchling *Chrysemys picta* (Costanzo et al. 2003).

Recent studies suggest that extended periods of supercooling or supercooling to very low temperatures may induce physiological stress and even cause mortality (Packard and Packard 1999; Hartley et al. 2000; Costanzo et al. 2001a). Our hatchling *G. geographica* readily tolerated a relatively brief exposure to −8 °C, although we do not know the thermal and temporal limits for survival in the supercooled state. Owing to diminished tissue perfusion, survival at such low temperatures may require a capacity for sustained anaerobic respiration as well as a tolerance for the decrease in pH associated with an accumulation of lactate (Hartley et al. 2000; Costanzo et al. 2001a). Our hatchling *G. geographica* accumulated lactate and glucose during supercooling, much as did the hatchling *Chrysemys picta* studied by Costanzo et al. (2001a), probably as a consequence of ischemic hypoxia. Nevertheless, our turtles readily tolerated environmental anoxia for 8 days and, whereas lactate concentrations in two of these hatchlings exceeded 25 μmol g^{-1}, levels in the remaining turtles still were within the range of control values. Thus, the demonstrated capacity for anoxia tolerance in hatchling *G. geographica* may promote survival in the supercooled state during ecologically relevant exposures to subfreezing temperatures.

Inoculation resistance

Cold-hardy ectotherms can exploit a high capacity for supercooling only if they remain free of agents that would catalyze the freezing of their body fluids. This is a particularly significant challenge confronting terrestrially hibernating turtles, because the soil surrounding them is rife with ice and various INA that can inhibit their ability to supercool (Costanzo et al. 2000a). Ice is probably the most serious threat, although INA, such as soil particles, dust, and ice-nucleating microorganisms, can also trigger the freezing of supercooled turtles (Costanzo et al. 2000a). Our finding of diminished supercooling capacity in hatchlings immersed in oven-dried soil, which lacked ice crystals, is evidence of the deleterious effect of soil-borne INA.

The strong dependence of inoculation resistance in hatchling *G. geographica* on soil moisture level accords with earlier findings for hatchling *Chrysemys picta* (Costanzo et al. 1998, 2001c). Soil moisture is an important factor because it influences the probability that a hatchling will come into contact with ice. For the same reason, susceptibility to inoculative freezing is influenced by characteristics of the soil that affect particle size and porosity, and its ability to adsorb moisture (Costanzo et al. 1998, 2001c). Meaningful assessments of a species' capacity for inoculation resistance must be based on tests that use native substrata and ecologically appropriate moisture levels. Nevertheless, such experiments cannot perfectly replicate conditions within the nest and thus their results would not necessarily predict freezing risk or mortality in wild populations. Our inoculation resistance trial is conservative in that it exposes turtles to ice and INA with a maximal degree of intimacy; whereas, in natural nests, the presence of eggshells, air pockets, and other turtles may reduce the degree of exposure. This may explain why we found no overwintering mortality, even among turtles that were exposed to environmental conditions that induced freezing of hatchlings in our laboratory tests (Fig. 2).

How does the capacity for inoculation resistance in hatchling *G. geographica* compare to that of other species? When we tested additional hatchling *G. geographica* under conditions (loamy sand/clay mixture, 0.075 g water g^{-1} soil, water potential = −400 kPa) identical to those used in an earlier, comparative study (Costanzo et al. 2001b), our hatchlings resisted inoculation until they had cooled to nearly −10 °C (mean ± SEM = −9.7 ± 1.0 °C; $n = 7$). This measure of performance is second only to that reported for *Chrysemys picta* (−13.6 ± 0.9 °C; $n = 5$) and far exceeded that determined for three species of aquatic hibernators and four other taxa of terrestrial hibernators (Costanzo et al. 2001b).

Substantial taxonomic differences in inoculation resistance among hatchling turtles may reflect morphological variation with respect to the expanse of skin exposed to the environment (Costanzo et al. 2001b) as well as differences in the ultrastructure of the integument (Willard et al. 2000). The superior inoculation resistance of hatchling *Chrysemys picta* was attributed to the presence of large deposits of unsaturated lipids in the dermal and epidermal layers of skin that is chronically

exposed to the environment (Willard et al. 2000). Whether hatchling *G. geographica* also possess integumental lipid deposits remains to be determined. Nevertheless, our finding that hatchling *G. geographica* are exceptionally resistant to both inoculation and dehydration bolsters the hypothesis that a similar morphological attribute governs both properties and suggests that the integument plays a major role in adaptation to terrestrial hibernation (Costanzo et al. 2001b).

In summary, we found that hatchling *G. geographica* routinely overwinter within the natal nest. These turtles are well adapted to terrestrial hibernation, as they are highly resistant to dehydration and can tolerate subfreezing temperatures. They lack freeze tolerance, but rather exploit a strategy of freeze avoidance, via supercooling, that depends on their exceptional resistance to inoculation by ice and INA within the nest microenvironment, and, perhaps, selection of relatively dry nesting sites. The well-developed cold hardiness of hatchling *G. geographica* may explain, in part, the historical extension of this species' range into southern Canada.

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