Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology

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Source: *Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches*, Vol. 77, No. 1 (January/February 2004), pp. 74-99
Published by: The University of Chicago Press . Sponsored by the Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology
Stable URL: http://www.jstor.org/stable/10.1086/378141
Accessed: 17-03-2016 14:04 UTC

Physiological Ecology of Overwintering in the Hatchling Painted Turtle: Multiple-Scale Variation in Response to Environmental Stress

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Accepted 5/5/03

ABSTRACT

We integrated field and laboratory studies in an investigation of water balance, energy use, and mechanisms of cold-hardiness in hatchling painted turtles (*Chrysemys picta*) indigenous to west-central Nebraska (*Chrysemys picta bellii*) and northern Indiana (*Chrysemys picta marginata*) during the winters of 1999–2000 and 2000–2001. We examined 184 nests, 80 of which provided the hatchlings ($n = 580$) and/or samples of soil used in laboratory analyses. Whereas winter 1999–2000 was relatively dry and mild, the following winter was wet and cold; serendipitously, the contrast illuminated a marked plasticity in physiological response to environmental stress. Physiological and cold-hardiness responses of turtles also varied between study locales, largely owing to differences in precipitation and edaphics and the lower prevailing and minimum nest temperatures (to −13.2°C) encountered by Nebraska turtles. In Nebraska, winter mortality occurred within 12.5% (1999–2000) and 42.3% (2000–2001) of the sampled nests; no turtles died in the Indiana nests. Laboratory studies of the mechanisms of cold-hardiness used by hatchling *C. picta* showed that resistance to inoculative freezing and capacity for freeze tolerance increased as winter approached. However, the level of inoculation resistance strongly depended on the physical characteristics of nest soil, as well as its moisture content, which varied seasonally. Risk of inoculative freezing (and mortality) was greatest in midwinter when nest temperatures were lowest and soil moisture and activity of constituent organic ice nuclei were highest. Water balance in overwintering hatchlings was closely linked to dynamics of precipitation and soil moisture, whereas energy use and the size of the energy reserve available to hatchlings in spring depended on the winter thermal regime. Acute chilling resulted in hyperglycemia and hyperlactemia, which persisted throughout winter; this response may be cryoprotective. Some physiological characteristics and cold-hardiness attributes varied between years, between study sites, among nests at the same site, and among siblings sharing nests. Such variation may reflect adaptive phenotypic plasticity, maternal or paternal influence on an individual's response to environmental challenge, or a combination of these factors. Some evidence suggests that life-history traits, such as clutch size and body size, have been shaped by constraints imposed by the harsh winter environment.

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Physiological and Biochemical Zoology 77(1):74–99. 2004. 1522-2152/2004/7701-2177$15.00

Introduction

The painted turtle (*Chrysemys picta*) is a common resident of freshwater ponds, marshes, and streams and is broadly distributed across temperate North America. This species is cold-hardy and anoxia tolerant and occurs farther north than any other New World chelonian (Ultsch 1989; Holman and Andrews 1994).

Hatching occurs in late summer, but neonates commonly remain in their nests until the following spring. Because their nests are shallow (5–10 cm) and are constructed in open terrain, these hatchlings may be challenged by environmental temperatures that fall below the equilibrium freezing point of their tissues, approximately −0.6°C. In regions where winters are cold and insulating snow cover is sparse or ephemeral (e.g., the upper Great Plains), nest temperatures can descend several degrees below this mark (Costanzo et al. 1995; Packard et al. 1997). In the Great Lakes region, snowfall is heavier and nest temperatures are more moderate, although occasionally hatchlings encounter subfreezing temperatures (Breitenbach et al. 1984; Nagle et al. 2000). In northern populations, winter survival of hatchling *C. picta* ranges from very high (Breitenbach et al. 1984) to nil (St. Clair and Gregory 1990) and probably varies with winter severity, other climatological factors, and edaphics.

Seminal studies of the cold-hardiness mechanisms of hatch-

ling *C. picta* are now more than a decade old, and dozens of reports have appeared subsequently (see reviews: Lee and Costanzo 1998; Packard and Packard 2001*b*). This body of work has established that hatchlings can survive chilling either by supercooling (remaining in the metastable, unfrozen state) or by tolerating somatic freezing. The thermal minimum for freezing survival appears to be −3° to −4°C (Storey et al. 1988; Churchill and Storey 1992*b*; Costanzo et al. 1995; Packard et al. 1999*b*). Turtles can survive at much lower temperatures (e.g., −12°C) if they remain supercooled (Paukstis et al. 1989; Packard and Packard 1993, 1999; Costanzo et al. 1999), although doing so is not always possible because physical contact with ice crystals or ice-nucleating agents (INAs) found in nesting soil (e.g., soil particulates, organic compounds, and certain microorganisms; Costanzo et al. 2000*a*, 2003) can trigger ice nucleation in the tissues. Given that the risk of inoculative freezing is strongly influenced by moisture content, texture, and other characteristics of nesting soil (Packard and Packard 1997; Costanzo et al. 1998, 2001*c*), the cold-hardiness strategy of hatchling *C. picta* may depend on both freeze tolerance and supercooling (Ultsch 1989; Costanzo et al. 1995). However, no direct observations of hatchlings utilizing either strategy under natural conditions have been made.

Recent laboratory studies revealed that hatchling *C. picta* may undergo profound physiological changes in preparation for overwintering. For example, cold acclimation coincides with increases in plasma osmolality, supercooling capacity, and resistance to inoculative freezing (Costanzo et al. 2000*b*; Packard et al. 2001) and also with elimination of INAs from the gut (Packard et al. 2001; Costanzo et al. 2003). Hatchlings mobilize the putative cryoprotectants glucose and lactate during extreme chilling, whether they remain supercooled (Hartley et al. 2000; Costanzo et al. 2001*a*) or they freeze (Storey et al. 1988; Churchill and Storey 1991, 1992*b*). Although such studies are instructive, a systematic investigation of the physiological ecology of hatchlings undergoing natural hibernation has not been undertaken, perhaps owing to the difficulty of sampling large numbers of turtles directly from their nests in winter.

Our study focused on hatchling painted turtles (*Chrysemys picta bellii*) indigenous to the Sandhills of west-central Nebraska. This site has been under investigation by one of us (J. B. Iverson) for over 21 yr and was the setting for our germinal study of cold-hardiness in an assemblage of northern turtles (Costanzo et al. 1995). We also studied hatchlings (*Chrysemys picta marginata*) at a nesting site in northern Indiana (Costanzo et al. 2001*c*). These locales are inhabited by some of the same species of turtles, but they differ markedly in edaphics, physiognomy, and climate. We anticipated that such differences would influence the winter physiological ecology of turtles native to these locales. In addition, we sought to investigate seasonal and year-to-year variation in hatchling physiology, as well as variation among nests at the same locale and among hatchlings sharing the same nest.

Material and Methods

Study Areas

Our study area in west-central Nebraska is located 45 km north of Oshkosh, Nebraska, and is centered near Gimlet Lake (41°N, 102°W) on the Crescent Lake National Wildlife Refuge (hereafter, CLNWR). The refuge encompasses ~18,615 ha of relatively undisturbed midgrass prairie interspersed with numerous water-table lakes and wetlands. The climate in this region is characterized by cold, dry winters; warm, wet springs; hot, dry summers; and cool, dry autumns. Snowfall is sparse, and commonly there is little snow cover to buffer soil from severely cold air. Indigenous western painted turtles (*Chrysemys picta bellii*) lay ~14 eggs in nests constructed in predominantly sandy soils, and the hatchlings overwinter at a depth of ~10 cm below the soil surface. Other turtles found at or near CLNWR include common snapping turtles (*Chelydra serpentina*), spiny softshelled turtles (*Apalone spinifera*), yellow mud turtles (*Kinosternon flavescens*), and ornate box turtles (*Terrapene ornata*).

Our study area in northern Indiana (41°N, 86°W) is located near Mount Zion Millpond (hereafter, MZMP) in Fulton County, 70 km south of South Bend, Indiana. This 20-ha pond was formed in the 1800s when Rain Creek, a minor tributary of the Tippecanoe River, was impounded to power a sawmill. Nesting occurs on a 1.2-ha area encompassing a residence and surrounding mowed lawn adjacent to the pond. The local climate is characterized by cold, wet winters; cool, wet springs; hot, humid summers; and cool, damp autumns. Snowfall can be heavy, and snow cover may persist for long periods. Indigenous midland painted turtles (*Chrysemys picta marginata*) lay approximately seven eggs in nests constructed in loamy soils, and the hatchlings overwinter at a depth of ~5 cm below the soil surface. Other turtles found at MZMP include *Chelydra serpentina*, *A. spinifera*, red-eared sliders (*Trachemys scripta elegans*), Blanding's turtles (*Emydoidea blandingii*), northern map turtles (*Graptemys geographica*), stinkpots (*Sternotherus odoratus*), and eastern box turtles (*Terrapene carolina*).

Sampling Regimen

During late May through early June 1999 and 2000, we located a total of 232 nests by observing nesting females. After a spent turtle retreated, we marked and mapped its nest and covered it with wire screen (~400 cm^2) to deter predation. In 1999, we marked and protected 51 CLNWR nests and 50 MZMP nests. In 2000, we marked and protected 83 and 48 nests at these sites, respectively. Approximately 90% of the MZMP nests were produced by turtles making their initial nesting effort of the season, whereas the CLNWR samples included roughly equal numbers of nests made during first and second nesting efforts.

We placed single-channel temperature loggers (Tidbit, Onset Computer, Pocasset, Mass.) inside some nests. These units were deployed during oviposition and placed among eggs (CLNWR)

or were carefully inserted into the nest chamber via a small tunnel in November (MZMP). We used additional loggers to record air temperature and temperature of the soil at the usual depth of the nest chamber (CLNWR, 10 cm; MZMP, 5 cm) at representative locales at each study site and to monitor the depth-temperature profile in the soil column at four permanent stations (MZMP only). These stations were outfitted with dataloggers (Stowaway, Onset Computer) whose thermistor probes were positioned at depths of 5, 10, 15, and 20 cm. All loggers recorded temperature hourly for the duration of the study period. We measured precipitation using tipping-bucket rain gauges that were outfitted with event loggers (Onset Computer) and/or obtained records from weather stations ≤0.1 km (CLNWR) or 8.5 km (MZMP) distant.

We collected hatchlings and samples of nest soil from some of the marked nests on three to five occasions in 1999–2000 and in 2000–2001. Initial and final sample dates were intended to correspond with the onset of sustained cold and with incipient nest emergence associated with the termination of winter dormancy, respectively. Interim samples were taken opportunistically, when weather conditions were conducive to excavating nests. Because a few of the sampled nests contained temperature loggers, we were able to associate thermal history with physiological condition for some hatchlings. We sampled CLNWR nests on October 2 ($n = 5$), January 17 ($n = 9$), February 27 ($n = 3$), and April 8 ($n = 5$) in 1999–2000 and on September 30 ($n = 8$), December 19 ($n = 6$), February 4 ($n = 7$), March 4 ($n = 3$), and April 7 ($n = 6$) in 2000–2001. We sampled MZMP nests on November 17 ($n = 4$), February 16 ($n = 4$), and March 23 ($n = 6$) in 1990–2000 and on November 27 ($n = 5$), January 17 ($n = 5$), and April 2 ($n = 4$) in 2000–2001. In addition, samples of soil were collected, at a depth of 5 cm, from each of the four permanent MZMP stations in both 1999–2000 (December 1, January 9, February 16, March 8, and March 23) and 2000–2001 (January 17 and April 2).

At sampling time, we carefully excavated the nest with a spoon after clearing away any vegetative detritus or snow that might infiltrate the exposed nest chamber. Hatchlings, along with any unhatched eggs and eggshell fragments, were quickly transferred to a plastic canister and covered with soil taken from the nest chamber. The canister was closed with a plastic lid that inhibited drying of the soil, although gas exchange was permitted via a few small holes in the lid. Our aim was to expose the hatchlings, as much as possible, to conditions similar to those in the nest chamber. A sample of soil taken from each nest chamber was placed in a moisture-proof glass vial, which, together with the canisters and ice packs, was placed in an insulated padded box and shipped to our laboratory by express courier. A temperature logger placed adjacent to the canisters confirmed that the samples remained cool (~4°C) during the 24–48-h transit from the field to our laboratory. On arrival, we placed the samples in an incubator (4°C) and immediately began our analyses. Experimental procedures involving turtles were approved by the Animal Care and Use Committee of Miami University (protocol 429).

We excavated 103 nests in addition to those providing material for laboratory analyses. Some were intended for this purpose but were discovered to be devoid of hatchlings. Most, however, were examined in the spring in order to assess winter mortality. We discerned the fate of any unhatched eggs (early- vs. late-term mortality) and the time of death (summer vs. winter) of any dead hatchlings. Although no marked CLNWR nests remained in spring 2000, we examined 22 CLNWR nests on April 7, 2001. At MZMP, spring samples consisted of 25 and 24 nests in 2000 and 2001, respectively. Some of these nests contained temperature loggers, permitting us to evaluate the relationship between the thermal regime and winter mortality.

Somatic Characteristics

Turtles were removed from the chilled shipping canisters, gently brushed to remove adherent soil, weighed to 0.01 g, and measured to 0.1 mm with a caliper to determine carapace and plastron lengths. They were killed by decapitation and their carcasses were weighed to the nearest 0.1 mg and then dried to constant mass in a 65°C oven for ~1 wk. They were again weighed, and body water content was calculated from the water lost on drying.

Dried carcasses were finely ground in a coffee mill. A 100-mg sample of the homogenized material was analyzed for nonpolar lipids using a methanol/chloroform extraction procedure (Tietz 1970). An additional sample (~500 mg) was placed in a porcelain crucible and incinerated at 550°C in a muffle furnace. The difference in mass of the sample before and after incineration was used to calculate the organic content of the carcass.

We measured morphometric variables, as well as carcass water, lipid, and organic contents, for all turtles examined in the study. However, values for the turtles that were found dead in their nests were excluded from the primary statistical analyses.

Blood Sampling and Metabolite Analyses

We collected blood from up to five live turtles in each nest for use in hematological and metabolite analyses. Turtles were cleaned by swabbing skin of the head and neck with ethyl alcohol and were killed by severing the spinal cord from the cranium with a scissors, using care to avoid lacerating the trachea and esophagus. Blood was drawn from severed neck vessels into heparinized microcapillary tubes, which were centrifuged (2,000 *g*, 5 min) in order to pack the erythrocytes. We measured the hematocrit and harvested ~70 μL of plasma from each turtle. Blood plasma was stored frozen (−80°C) until used.

Plasma osmolality was measured using a vapor pressure osmometer (Wescor, model 5500, Logan, Utah) and NaCl standards. Enzymatic assays were used to determine plasma con-

centrations of glucose (Sigma, no. 510, St. Louis), glycerol (Sigma, no. 337), and lactate (Sigma, no. 735). Urea nitrogen in plasma was measured using a urease/nitroprusside method (Sigma, no. 640). Total protein was measured using the Bradford procedure (BioRad, Richmond, Calif.) with bovine serum albumin as the standard. In order to extend the sample, these analyses used plasma diluted (1 : 1) with ultrapurified water (Costanzo et al. 2000*b*).

Cold-Hardiness Trials

When available, additional live turtles were subjected to cold-hardiness trials. We determined the level of inoculation resistance by measuring the temperature of crystallization (T_c) of turtles cooled while immersed in a matrix of frozen nest soil (Costanzo et al. 1998). Turtles were placed individually in 50-mL plastic tubes and enveloped in ~12 g of soil collected from

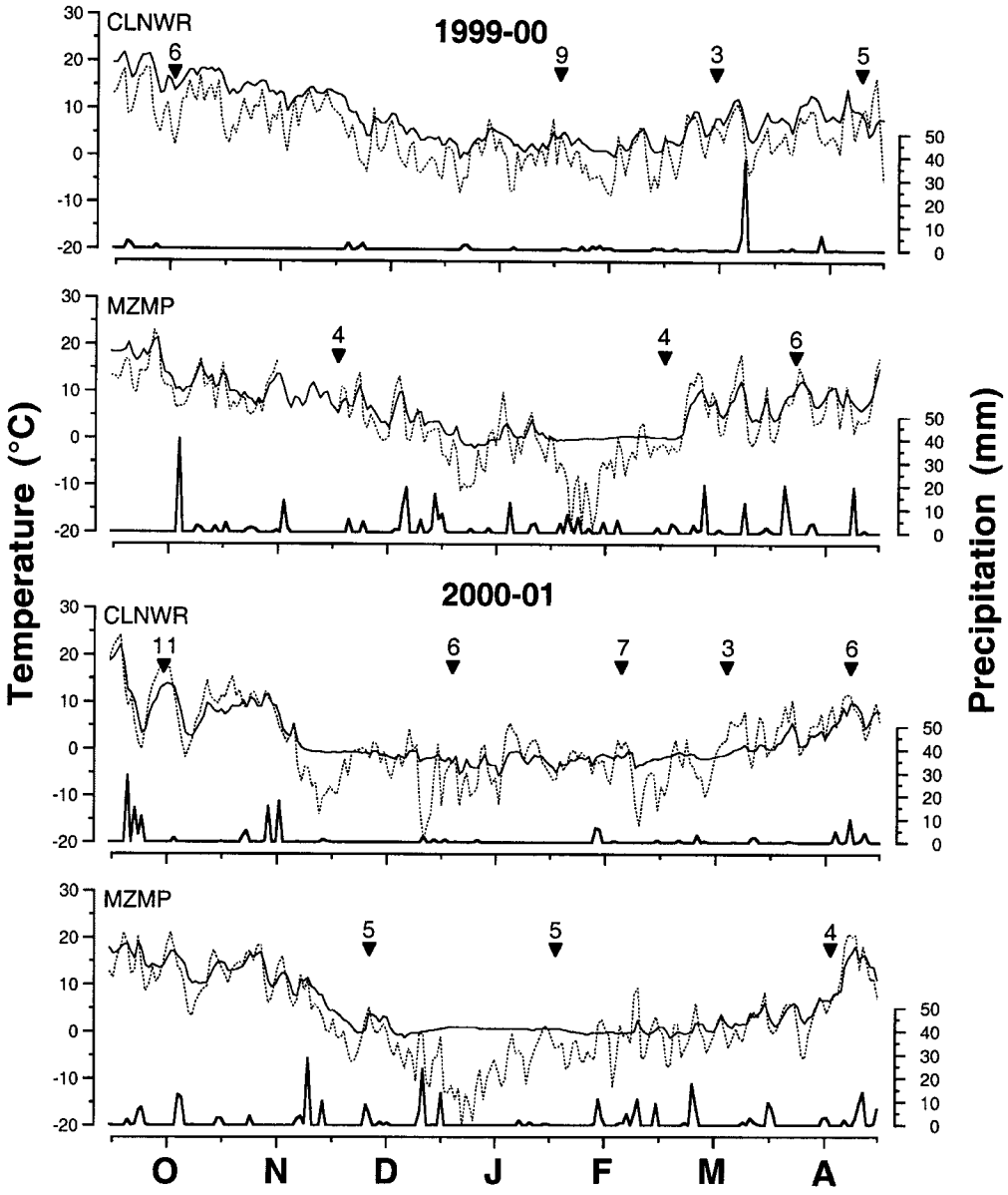

Figure 1. Precipitation (*bold line*), average daily air temperature (*dotted line*), and average daily soil temperature (*thin solid line*) at study areas in west-central Nebraska (CLNWR) and northern Indiana (MZMP) during 2 yr of study. Soil temperatures were recorded at the typical depth of the nest chamber (CLNWR, 10 cm; MZMP, 5 cm). Arrows indicate times when hatchlings and samples of soil were collected from nests; numeral above each arrow indicates number of nests sampled.

their respective nests. The soil was carefully tamped around the turtle in a manner that promoted uniform, intimate contact with external INAs. A plastic foam plug was placed above the soil and turtle in order to increase thermal stability of the system and to aid retention (and detection) of the turtle exotherm. Tubes were suspended in a refrigerated ethanol bath (Neslab model RTE 140, Portsmouth, N.H.) set at −0.4°C, bringing their contents to an equilibrium temperature slightly below 0°C. The soil was inoculated with small ice crystals and permitted to freeze for 1 h, after which the turtle, then completely enveloped in frozen soil, was chilled at 0.5°C h^{-1} until it produced a freezing exotherm. During cooling, temperature, as registered by a thermocouple held against the carapace, was logged at 30-s intervals on a chart recorder (Omega model RD3752, Stamford, Conn.).

In freeze-tolerance trials, we determined the survival status of turtles subjected to a standardized episode of somatic freezing. Turtles were placed in small groups inside a plastic canister and immersed in chilled soil collected at the nesting site. To these artificial nests we added water as needed to attain a soil moisture level of 10% (w/w dry mass). This technique ensured that turtle tissues would nucleate without supercooling extensively (Costanzo et al. 1998), a condition requisite for freezing survival (Storey and Storey 1988).

During the trial, soil temperature, measured using a thermocouple placed among the turtles, was logged at 30-s intervals on a chart recorder. We chilled turtles to approximately −0.4°C by placing the canisters in a large beaker that was submerged in a refrigerated alcohol bath (Forma Scientific, Marietta, Ohio). After inoculating the soil with ice, we further cooled the bath until the soil and turtles attained an equilibrium temperature of −3°C. Turtles remained at this temperature for 72 h and then were slowly warmed to 0°C. Although our testing protocol was designed to ensure that freezing would begin at a temperature near −0.6°C, we nevertheless inspected the turtles before they had fully thawed in order to confirm that they had frozen. We determined survival status on the basis of whether turtles responded to tactile stimulation and were capable of locomotion after being warmed to room temperature.

Analysis of Nest Soils

We determined the moisture content and ice-nucleating activity of organic INAs in samples of nest soil because these factors strongly influence the ability of turtles to remain supercooled within their nests (Costanzo et al. 1998, 2000*a*). Duplicate portions of the soil samples shipped to the laboratory in sealed vials were weighed to 0.1 mg, dried to constant mass in a 65°C oven, and then reweighed. Moisture content was calculated from the change in mass, and the results were averaged to produce a single value representing each nest.

Another portion of the soil sample was used in a standardized procedure that gauges potency of constituent organic INAs (Costanzo et al. 1998). We prepared a soil washing by combining 0.5 g soil and 1.0 mL autoclaved, ultrapurified water, vortexing the mixture for 60 s, and then sedimenting coarse particles by centrifugation (180 *g*, 3 min). The resultant supernatant was expressed through a 5-μm filter, and 10-μL aliquots of the filtrate were then drawn into the center of 20-μL glass capillary tubes whose ends were then sealed with clay. We determined ice-nucleating activity in these samples ($n = 6$ per washing) by cooling them (~1.5°C min^{-1}) in a refrigerated bath until each one froze. Freezing exotherms were detected by a thermocouple taped to the outside of each tube. For comparison, we also determined T_c values for samples of ultrapurified water, which lack potent INAs. Water, filters, utensils, and vessels used in preparing the washings were autoclaved before use in order to prevent contamination of the washing with exogenous INAs.

Statistical Inferences

We separately analyzed the data collected at each study site in 1999–2000 and 2000–2001. Seasonal variation in moisture content and INA activity in soil samples were tested using ANOVA, followed by Bonferroni multiple-comparisons tests. Somatic characteristics, physiological variables, and level of inoculation resistance determined for individual turtles were compared across multiple scales. In order to test variation among sample dates, we used nested ANOVAs with nest within sample date as a random factor. Least squares means and standard errors were contrasted using Bonferroni multiple comparisons. Separate ANOVAs were used to test for variation among the nests examined on each sample date. Somatic and physiological variation among turtles collected from the same nest was gauged from the coefficient of variation computed for each of the measured attributes. We used PROC CORR (SAS, version 8, SAS Institute, Cary, N.C.) to compute Pearson correlation coefficients for all pairings of somatic and physiological variables and to test the significance of those correlations (Hatcher and Stepanski 1994). Any analyses involving percentage data were performed on arcsine–square root transformed values. Significance was accepted at $P < 0.05$.

Results

Macro- and Microenvironmental Conditions at the Study Areas

Winter 1999–2000 at our study areas was relatively mild. Air temperatures at CLNWR and MZMP generally were similar; however, the prevailing soil temperature at the depth of turtle nests was 3°–5°C lower at MZMP (Fig. 1). Minimum soil temperature was −1.7°C at CLNWR and −3.4°C at MZMP.

In the second season of study, cold weather persisted from mid-November until March at both study areas, although conditions were more severe at CLNWR. With few exceptions, monthly average air temperatures were lower at CLNWR. Also,

Table 1: Survival of hatchling painted turtles at a study area in west-central Nebraska (CLNWR) during winter 2000–2001, in relation to nest minimum temperature and critical exposure duration, the period of time nest temperature was below the approximate equilibrium freezing point of turtle tissues, −0.6°C

Nest	Min. Temp. (°C)	Critical Exposure Duration (h)		No. Hatchlings	Winter Survival (%)
		Longest Bout	Winter Total		
31	−3.9	258	921	9	100
27	−4.2	69	716	12	100
10	−4.5	453	1,231	12	100
30	−5.7	141	860	8	75.0
18	−6.7	301	1,826	8	62.5
34	−6.8	715	2,340	0[a]	
26[b]	−7.1	645	2,281	13	30.8
22	−8.2	168	1,389	3	66.7
5	−9.4	620	2,328	0[c]	…
6[b]	−10.1	752	2,700	10	100
36	−10.2	287	2,089	10	0
15	−10.5	2,775	2,811	0[c]	…
1	−13.2	2,912	3,268	0[a]	…

Note. Survival was independent of minimum nest temperature ($r^2 = 0.33$, $F_{1,7} = 3.5$, $P = 0.10$) and critical exposure duration (single longest chilling bout: $r^2 = 0.002$, $F_{1,7} = 0.02$, $P = 0.90$; winter cumulative total: $r^2 = 0.21$, $F_{1,7} = 1.8$, $P = 0.22$). Minimum nest temperature was strongly correlated with critical exposure duration (single longest chilling bout: $r^2 = 0.51$, $F_{1,11} = 11.3$, $P = 0.010$; winter cumulative total: $r^2 = 0.77$, $F_{1,11} = 37.2$, $P < 0.0001$).

[a] Embryos failed to develop.

[b] Hatchlings were used in the April 7, 2001, sample.

[c] Predated before hatching.

at CLNWR, soil temperature at nest depth fell below −2°C each month from November through March (seasonal minimum, −7.1°C) and never rose above 0°C during December and January, whereas soil temperature at MZMP infrequently fell below 0°C, and the seasonal minimum temperature, recorded in February, was −3.0°C. The buffering effect of a persistent snow cover, which is evident in the data presented in Figure 1, moderated soil temperature at MZMP.

Temperature loggers placed inside actual turtle nests showed that at the usual time of hatching (late summer), nest temperature typically varied from 20° to 30°C on a daily basis. Average daily temperature and amplitude of the diel cycle decreased slightly from August through September, and then more precipitously in October. During winter, nest temperatures remained low (typically <10°C) and transiently fell below freezing on several occasions. Thermal minima to which hatchlings were exposed in 1999–2000 were −0.8°C at CLNWR ($n = 1$) and from −2.3° to −4.7°C at MZMP ($n = 4$). In 2000–2001, seasonal thermal minima were markedly lower in CLNWR nests (−3.9° to −13.2°C; $n = 13$; Table 1), and those in MZMP nests (−1.2° to −4.2°C; $n = 7$) were similar to those recorded the previous winter. Hatchlings in CLNWR nests were exposed to protracted periods of subfreezing cold during 2000–2001, as the cumulative time that soil temperature was below the equilibrium freezing point of turtle tissues, −0.6°C, ranged from 716 to 3,268 h (mean ± SD = 1,905 ± 819 h; $n = 13$ nests; Table 1). Owing to frequent snow cover, MZMP hatchlings spent relatively less time exposed to such temperatures (range: 6–727 h; mean ± SD = 155 ± 258 h; $n = 7$ nests). Generally, prevailing and minimum temperatures inside the instrumented MZMP nests were similar to those recorded in the soil column at a depth of 5 cm at the four permanent stations.

From October until March, precipitation was less frequent, and total precipitation was lower at CLNWR compared with MZMP (Fig. 1). At CLNWR, more precipitation fell during 2000–2001 (73 mm) than during 1999–2000 (24 mm). At MZMP, precipitation during this 5-mo interval did not differ appreciably between 2000–2001 (223 mm) and 1999–2000 (237 mm), although the second year of the study could be considered wetter if data from September 2000 were included, since 79 mm of rain fell during that month.

Moisture content of the soil inside *Chrysemys picta* nests did not vary seasonally in 1999–2000 (CLNWR: $F_{3,18} = 1.05$, $P = 0.39$; MZMP: $F_{2,11} = 1.90$, $P = 0.20$; Fig. 2). In 2000–2001, owing to an abundance of precipitation (Fig. 1), soil moisture levels remained high throughout autumn and winter (CLNWR: $F_{4,25} = 0.95$, $P = 0.45$; MZMP: $F_{2,11} = 0.33$, $P = 0.72$; Fig. 2). MZMP soil tended to be wetter than CLNWR soil, probably owing to its higher colloidal content (Costanzo et al. 2001*c*) and the greater precipitation in this region (Fig. 1). In each year of the study and at each study area, we found marked variation in soil moisture among the nests sampled on a given day. Overall, coefficients of variation in soil moisture computed for each sample day ranged from 0.12 to 0.80 for CLNWR nests and from 0.22 to 0.91 for MZMP nests.

Ice-nucleation temperatures of washings prepared from samples of nest soil collected in 1999–2000 and 2000–2001 ranged from −5.5° to −3.5°C for CLNWR nests and from −5.2° to −3.0°C for MZMP nests (Fig. 2). In contrast, samples of the ultrapurified water used to prepare washings froze at temperatures near −20°C. Ice-nucleating activity was independent of study area and year of study but tended to be highest in soil samples collected during the coldest segment of the study period (December–February; Fig. 2); however, the apparent seasonal variation lacked statistical significance in 1999–2000 (CLNWR: $F_{3,18} = 1.42$, $P = 0.27$; MZMP: $F_{2,11} = 0.57$, $P = 0.58$) and 2000–2001 (CLNWR: $F_{4,25} = 0.23$, $P = 0.92$; MZMP: $F_{2,11} = 1.02$, $P = 0.39$). We found pronounced variation in INA activity among the nests sampled on a given day, as T_c values for individual nests differed by as much as 2.0°C.

Combining the data for all 80 turtle nests revealed a direct association between soil moisture content and INA activity

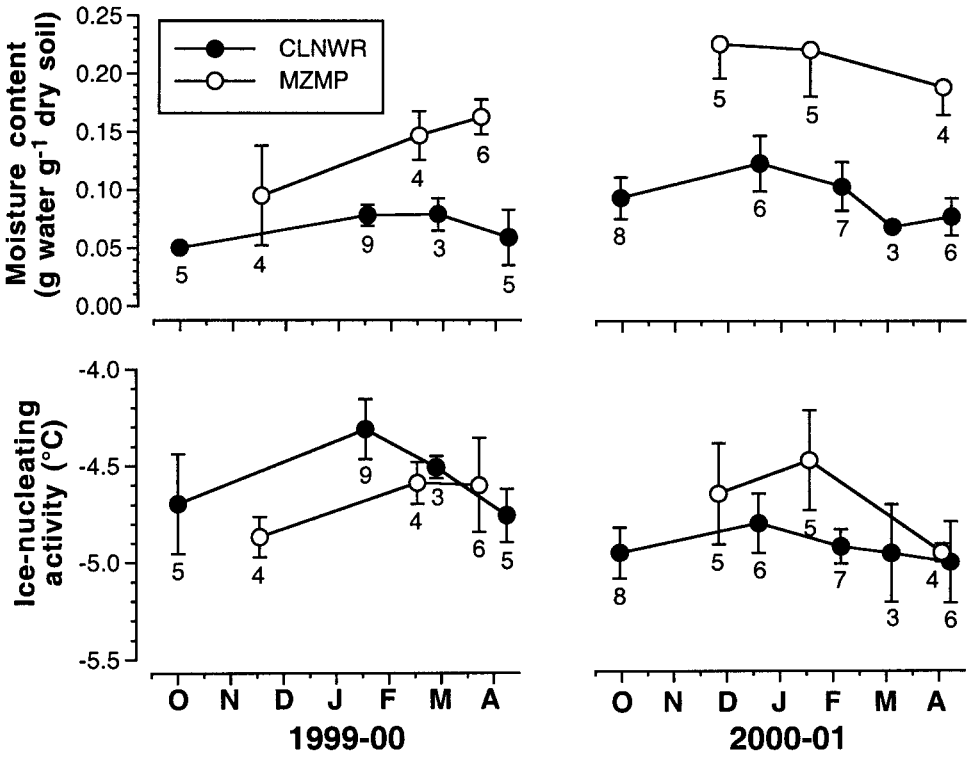

Figure 2. Moisture content and activity of organic INAs in samples of soil collected from chambers of painted turtle nests at study areas in west-central Nebraska (CLNWR) and northern Indiana (MZMP) during 2 yr of study. Means (±1 SE) are based on samples collected from the indicated number of nests.

($F_{1,78} = 11.2$, $P = 0.001$, $r^2 = 0.13$). Partitioning the data by study area and by study season showed that higher INA activity was associated with wetter soils in 2000–2001 (CLNWR: $F_{1,28} = 5.18$, $P = 0.031$, $r^2 = 0.16$; MZMP: $F_{1,12} = 5.46$, $P = 0.038$, $r^2 = 0.31$) but not in 1999–2000 (CLNWR: $F_{1,20} = 0.05$, $P = 0.83$, $r^2 = 0.002$; MZMP: $F_{1,12} = 3.24$, $P = 0.097$, $r^2 = 0.21$).

Soil samples collected at the four MZMP permanent stations over the 2-yr study varied by location with respect to moisture content ($F_{3,23} = 5.43$, $P = 0.006$) but not INA activity ($F_{3,20} = 1.14$, $P = 0.36$). Generally, levels of soil moisture (mean ± SE = 0.13 ± 0.01 g water g^{-1} dry soil; $n = 27$) and INA activity (mean ± SE = −4.6°C ± 0.1°C; $n = 24$) in these samples were similar to those measured in samples of soil collected inside MZMP turtle nests (Fig. 2).

Nest Recovery and Winter Survival of Hatchling Turtles

Of the 134 marked CLNWR nests, 27 (20.1%) were destroyed by predators, despite the presence of the wire screens, and 13 (9.7%) could not be relocated. The contents of 52 nests were sent to the laboratory for analysis, whereas only survival status of the hatchlings was determined for the remaining 42 nests. Eggs inside one nest examined in October 1999 were in the process of hatching. Unpipped eggs collected from another nest on the same date ultimately hatched in the laboratory. These hatchlings were used in physiological studies, but the resulting data were excluded from the statistical analyses. In October 2000 we collected hatchlings that had recently emerged from a nest and were traveling abroad, presumably en route to Gimlet Lake. Data for these turtles were omitted from the statistical analyses.

We marked a total of 98 MZMP nests. Two of these nests were predated, and six nests could not be relocated; however, the remaining nests were sampled for use in laboratory analyses ($n = 28$) or examined to determine winter mortality ($n = 62$). Hatchlings from one nest emerged on September 5, 1999, although we collected neither these turtles nor a sample of soil from their nest. Unexpectedly, two intact eggs removed from a nest in January 2001 each contained a viable turtle that later hatched in the laboratory, despite being held at 4°C. This nest also contained a single neonate, which we subjected to metabolite analyses.

Except as noted above, nests held intact eggs containing dead embryos, live hatchlings, hatchlings that had died either before winter (partly decomposed, desiccated, dull plastral color) or

during winter (no odor, turgid, and bright plastral color), or a combination thereof. We found no winter-killed hatchlings at MZMP in either year of the study. At CLNWR winter mortality varied by year. In 1999–2000 we found one winter-killed turtle in a nest sampled in January and two in a nest sampled in February; however, none was found in the six nests examined in April. By contrast, substantial mortality occurred during the following winter, as 18 (64.3%) of the 28 nests we examined in April 2001 contained at least one winter-killed turtle, and five of these nests contained no survivors. In addition, we found winter-killed turtles in four of the five nests sampled in December and in three of the five nests sampled in February. The greater mortality in winter 2000–2001 coincided with lower soil temperatures (Fig. 1) and higher soil moisture levels (Fig. 2).

Seasonal Variation in Somatic Characteristics and Physiology

Inferences about seasonal changes in somatic characteristics and physiology of hatchlings were based on trends in the data for turtles collected over the course of winter. Because carapace length varied ($P < 0.05$) among the samples collected in 1999–2000 (CLNWR) and in 2000–2001 (CLNWR, MZMP), any variation in somatic and physiological characteristics might simply reflect differences in body size rather than a response to a seasonally dynamic microenvironment. Therefore, we reanalyzed the data using a nested analysis of covariance (ANCOVA) with carapace length as the potential covariate. Significant covariation was found for plastron length, fresh body mass, dry body mass, carcass water content, and plasma urea concentration; hence, for these variables, we report least squares means adjusted for differences in carapace length. Analyses of all other variables reverted to the basic nested ANOVA.

Most turtles weighed 4–5 g and measured ~24 mm in carapace length (Table 2). We found no marked differences in body mass or carapace length between *Chrysemys picta bellii* (CLNWR) or *Chrysemys picta marginata* (MZMP). Fresh body mass and dry body mass varied during winter, even after accounting for variation in carapace length, but without a discernible pattern (Table 2). This outcome suggests that turtles also varied in carapace shape and/or somatic density. Unexpectedly, the turtles did not lose mass (Table 2).

Seasonal changes in water balance were gauged from measurements of carcass water content, expressed as a percentage of fresh carcass mass, and plasma osmolality. Generally, carcass water content tended to decrease and osmolality tended to increase during winter 1999–2000, suggesting that CLNWR and MZMP turtles were under water stress. These measures also changed during winter 2000–2001 but in opposite directions: turtles tended to gain water, and plasma osmolality decreased (Table 2). Maintenance of a positive water balance may have been linked to the ample precipitation (Fig. 1) and soil moisture (Fig. 2) in the second winter of study.

Seasonal variation in carcass organic matter and lipid content (Table 2) was reflective of energy balance in overwintering turtles. During winter 1999–2000, CLNWR turtles exhibited no appreciable decrease in organic matter content but a slight (14%) reduction in lipid content, whereas MZMP turtles showed marked decreases in both variables (14% and 35%, respectively). Turtles expended less energy during winter 2000–2001, as any variation in carcass organic matter and lipid content was either slight or nonsignificant (Table 2). Furthermore, lipid concentration in MZMP turtles at the end of hibernation was twice as great in 2000–2001 than in 1999–2000 (Table 2). In these turtles, nonpolar lipid reserves were an important energy source during the mild winter of 1999–2000. However, given that lipid catabolism accounted for only 27% of the measured decrease in organic matter, turtles must have mobilized other energy-yielding substrates, such as glycogen and protein, to meet their energy demands.

Mean hematocrit ranged from 24% to 34%. Hematocrit varied slightly during winter in CLNWR turtles, generally being higher in 1999–2000 than in 2000–2001 (Table 2). We found no variation in hematocrit among MZMP turtles in either year of the study.

Overall, seasonal changes in the levels of key metabolites were similar between CLNWR and MZMP turtles (Table 2). With some metabolites, the pattern of change varied by year, probably in response to the particular hydric and thermal regimes prevailing within nests. Generally, the physiological state of the turtles sampled during the mild, dry winter of 1999–2000 reflected water stress, whereas that of the turtles sampled during the colder, relatively wet winter of 2000–2001 reflected hypothermic stress.

During winter, plasma urea levels either increased, albeit sometimes only transiently, or were unchanged (Table 2). For both CLNWR and MZMP turtles, urea concentrations were markedly greater in 1999–2000 than in 2000–2001, with the MZMP turtles sampled in mid-February exhibiting the highest levels (12–25 μmol mL^{-1}).

Plasma glucose levels increased substantially (up to 15-fold) in winter, the onset of hyperglycemia coinciding with the advent of frost. Plasma glucose concentrations decreased as winter proceeded but remained above those measured in autumn turtles (Table 2; Fig. 3). Plasma glucose levels tended to be higher in 2000–2001 and in CLNWR turtles, suggesting that glycemia was influenced by degree of cold exposure. Indeed, turtles exposed to severe chilling episodes were extremely hyperglycemic, with concentrations in some individuals reaching 60 μmol mL^{-1} (Fig. 3).

We found low concentrations (<0.5 μmol mL^{-1}) of glycerol in the blood of most turtles (Table 2). Glycerol levels varied during winter, but there was no marked accumulation signifying that the polyol was mobilized in response to chilling.

Mean plasma lactate concentrations in winter turtles were up to threefold higher than those measured in autumn turtles (Table 2). Nevertheless, except for a few individuals that had

Table 2: Somatic and physiological characteristics of hatchling painted turtles and dates sampled at study areas in west-central Nebraska (CLNWR) and northern Indiana (MZMP) during 2 yr of study

	10/1/99 (5 nests)	1/17/00 (2 nests)	2/27/00 (3 nests)	4/8/00 (5 nests)	*F*	df	*P*
CLNWR, 1999–2000:							
Carapace length (mm)	24.5 ± .1^{A}	24.5 ± .2^{A}	25.6 ± .2^{B}	25.1 ± .2^{AB}	12.7	3, 149	.0001
Plastron length (mm)[a]	23.7 ± .1^{A}	24.2 ± .2^{BC}	24.4 ± .1^{C}	23.9 ± .1^{AB}	8.3	3, 148	.0001
Fresh body mass (g)[a]	4.56 ± .04^{A}	4.28 ± .1^{B}	4.85 ± .1^{C}	4.59 ± .1^{A}	13.0	3, 148	.0001
Dry body mass (g)[a]	1.18 ± .01^{A}	1.15 ± .02^{A}	1.29 ± .02^{B}	1.17 ± .02^{A}	11.5	3, 145	.0001
Carcass water content (% fresh mass)[a]	74.2 ± .2^{AC}	73.1 ± .3^{B}	73.6 ± .2^{BC}	74.6 ± .3^{A}	5.2	3, 145	.0021
Plasma osmolality (mosmol kg^{-1})	318 ± 4^{A}	432 ± 7^{B}	401 ± 5^{C}	401 ± 6^{C}	99.4	3, 54	.0001
Carcass organic content (mg g^{-1} dry mass)	758 ± 6^{A}	832 ± 10^{B}	716 ± 7^{C}	813 ± 9^{B}	39.0	3, 148	.0001
Carcass lipid content (mg g^{-1} dry mass)	118 ± 1^{A}	114 ± 2^{A}	105 ± 2^{B}	102 ± 2^{B}	18.6	3, 148	.0001
Hematocrit (%)	29 ± 1^{A}	31 ± 1^{AB}	34 ± 1^{B}	33 ± 1^{AB}	4.8	3, 55	.0049
Plasma glucose (μmol mL^{-1})	4.2 ± .3^{A}	18.8 ± .5^{B}	8.3 ± .4^{C}	8.0 ± .4^{C}	242.2	3, 54	.0001
Plasma glycerol (μmol mL^{-1})	.16 ± .02^{A}	.24 ± .04^{AB}	.31 ± .03^{B}	.22 ± .03^{AB}	5.0	3, 54	.0038
Plasma lactate (μmol mL^{-1})	1.9 ± .3^{A}	2.3 ± .4^{AB}	3.6 ± .4^{B}	3.1 ± .4^{AB}	5.9	3, 53	.0014
Plasma urea (μmol mL^{-1})[a]	8.8 ± .3^{A}	10.7 ± .4^{B}	5.8 ± .4^{C}	8.6 ± .3^{A}	26.5	3, 53	.0001
Plasma protein (mg mL^{-1})	19.6 ± .6^{A}	23.1 ± .7^{B}	20.3 ± .6^{A}	18.4 ± .6^{A}	9.0	3, 54	.0001
	11/17/99 (4 nests)	2/16/00 (4 nests)	3/23/00 (3 nests)		*F*	df	*P*
MZMP, 1999–2000:							
Carapace length (mm)	24.8 ± .2^{A}	25.0 ± .3^{A}	24.4 ± .3^{A}		1.4	2, 45	.2570
Plastron length (mm)[a]	23.5 ± .1^{A}	23.7 ± .2^{A}	23.5 ± .2^{A}		.4	2, 44	.6771
Fresh body mass (g)[a]	3.80 ± .04^{A}	4.32 ± .1^{B}	4.37 ± .1^{B}		51.9	2, 44	.0001
Dry body mass (g)[a]	.86 ± .01^{A}	1.10 ± .02^{B}	1.13 ± .02^{B}		106.4	2, 44	.0001
Carcass water content (% fresh mass)[a]	77.6 ± .3^{A}	74.4 ± .4^{B}	74.0 ± .4^{B}		43.8	2, 44	.0001
Plasma osmolality (mosmol kg^{-1})	388 ± 8^{A}	446 ± 10^{B}	415 ± 10^{AB}		9.9	2, 28	.0006
Carcass organic content (mg g^{-1} dry mass)	837 ± 6^{A}	794 ± 10^{B}	722 ± 8^{C}		61.1	2, 45	.0001
Carcass lipid content (mg g^{-1} dry mass)	90 ± 2^{A}	78 ± 3^{B}	59 ± 2^{C}		53.2	2, 45	.0001
Hematocrit (%)	31 ± 1^{A}	32 ± 1^{A}	31 ± 1^{A}		.3	2, 28	.7149
Plasma glucose (μmol mL^{-1})	3.1 ± .5^{A}	16.8 ± .6^{B}	9.3 ± .6^{C}		146.6	2, 28	.0001
Plasma glycerol (μmol mL^{-1})	.18 ± .03^{A}	.19 ± .04^{A}	.28 ± .04^{A}		2.2	2, 27	.1291
Plasma lactate (μmol mL^{-1})	1.9 ± .8^{A}	5.8 ± .9^{B}	2.7 ± .9^{AB}		5.4	2, 28	.0107
Plasma urea (μmol mL^{-1})[a]	13.8 ± .7^{A}	18.6 ± .9^{B}	12.2 ± .9^{A}		13.4	2, 27	.0001
Plasma protein (mg mL^{-1})	16.9 ± .9^{A}	22.3 ± 1.1^{B}	17.6 ± 1.0^{A}		8.2	2, 28	.0016

	9/30/00 (6 nests)	12/19/00 (5 nests)	2/4/01 (3 nests)	3/4/01 (3 nests)	4/7/01 (6 nests)	*F*	df	*P*
CLNWR, 2000–2001:								
Carapace length (mm)	23.3 ± .1^{A}	23.8 ± .1^{B}	23.9 ± .2^{ABC}	23.8 ± .1^{B}	24.3 ± .1^{C}	11.1	4, 204	.0001
Plastron length (mm)[a]	22.9 ± .1^{AC}	23.4 ± .1^{B}	22.5 ± .1^{C}	23.4 ± .1^{B}	23.3 ± .1^{AB}	10.9	4, 203	.0001
Fresh body mass (g)[a]	4.45 ± .1^{A}	4.40 ± .1^{A}	4.24 ± .1^{AB}	4.20 ± .1^{B}	4.30 ± .04^{AB}	4.1	4, 203	.0030
Dry body mass (g)[a]	1.22 ± .03^{A}	1.17 ± .02^{AB}	1.07 ± .03^{BC}	1.04 ± .02^{C}	1.13 ± .02^{B}	9.8	4, 191	.0001
Carcass water content (% fresh mass)[a]	72.7 ± .4^{A}	73.8 ± .3^{AB}	75.0 ± .4^{BC}	75.5 ± .3^{C}	74.0 ± .3^{AB}	8.8	4, 187	.0001
Plasma osmolality (mosmol kg^{-1})	378 ± 4^{A}	338 ± 4^{B}	342 ± 5^{B}	318 ± 5^{C}	321 ± 3^{C}	37.2	4, 77	.0001
Carcass organic content (mg g^{-1} dry mass)	747 ± 7^{A}	721 ± 7^{AB}	690 ± 9^{B}	703 ± 7^{B}	705 ± 5^{B}	9.7	4, 191	.0001
Carcass lipid content (mg g^{-1} dry mass)	109 ± 2^{A}	122 ± 2^{B}	102 ± 3^{A}	101 ± 2^{A}	104 ± 2^{A}	15.8	4, 193	.0001
Hematocrit (%)	26 ± 1^{AB}	28 ± 1^{BC}	24 ± 1^{A}	25 ± 1^{A}	29 ± 1^{C}	8.7	4, 77	.0001
Plasma glucose (μmol mL^{-1})	2.9 ± .9^{A}	43.1 ± .9^{B}	34.6 ± 1.2^{C}	26.3 ± 1.1^{D}	19.3 ± .8^{E}	278.3	4, 77	.0001
Plasma glycerol (μmol mL^{-1})	.25 ± .03^{A}	.22 ± .03^{A}	.04 ± .04^{B}	.04 ± .04^{B}	.13 ± .03^{AB}	8.1	4, 77	.0001
Plasma lactate (μmol mL^{-1})	1.5 ± .3^{AC}	3.6 ± .4^{B}	1.9 ± .5^{C}	4.1 ± .4^{B}	1.3 ± .3^{A}	12.6	4, 77	.0001
Plasma urea (μmol mL^{-1})[a]	5.4 ± .4^{A}	8.2 ± .4^{B}	8.7 ± .4^{BC}	8.3 ± .4^{BC}	9.7 ± .3^{C}	19.1	4, 76	.0001
Plasma protein (mg mL^{-1})	12.1 ± .5^{A}	16.5 ± .5^{B}	13.5 ± .6^{AC}	15.2 ± .6^{BC}	15.9 ± .4^{B}	13.4	4, 77	.0001

	11/26/00 (5 nests)	1/17/01 (5 nests)	4/2/01 (4 nests)	*F*	df	*P*
MZMP, 2000–2001:						
Carapace length (mm)	24.6 ± .3^{A}	24.4 ± .3^{A}	26.2 ± .2^{B}	20.6	2, 64	.0001
Plastron length (mm)[a]	24.1 ± .2^{A}	23.8 ± .2^{AB}	23.4 ± .1^{B}	3.3	2, 63	.0427
Fresh body mass (g)[a]	4.73 ± .1^{A}	4.71 ± .1^{A}	4.64 ± .1^{A}	.5	2, 63	.6418
Dry body mass (g)[a]	1.41 ± .03^{A}	1.17 ± .03^{B}	1.25 ± .02^{B}	29.4	2, 63	.0001
Carcass water content (% fresh mass)[a]	70.0 ± .4^{A}	75.5 ± .4^{B}	72.9 ± .2^{C}	71.5	2, 63	.0001
Plasma osmolality (mosmol kg^{-1})	317 ± 6^{AB}	327 ± 6^{A}	305 ± 5^{B}	3.7	2, 37	.0332
Carcass organic content (mg g^{-1} dry mass)	753 ± 8^{AB}	43 ± 9^{B}	774 ± 5^{A}	5.3	2, 54	.0082
Carcass lipid (mg g^{-1} dry mass)	114 ± 3^{A}	101 ± 3^{B}	118 ± 2^{A}	9.9	2, 58	.0002
Hematocrit (%)	34 ± 1^{A}	32 ± 1^{A}	32 ± 1^{A}	2.2	2, 37	.1239
Plasma glucose (μmol mL^{-1})	8.1 ± .6^{A}	10.5 ± .6^{B}	7.5 ± .5^{A}	7.5	2, 37	.0019
Plasma glycerol (μmol mL^{-1})	.14 ± .04^{A}	.35 ± .04^{B}	.14 ± .03^{A}	9.1	2, 37	.0006
Plasma lactate (μmol mL^{-1})	1.8 ± .5^{A}	2.9 ± .5^{A}	3.0 ± .4^{A}	2.4	2, 37	.1084
Plasma urea (μmol mL^{-1})[a]	11.3 ± .8^{A}	10.5 ± .8^{A}	11.2 ± .6^{A}	.4	2, 36	.6799
Plasma protein (mg mL^{-1})	23.6 ± .8^{A}	18.5 ± .7^{B}	16.8 ± .6^{B}	27.6	2, 37	.0001

Note. Values are least squares means ± SE based on the total number of turtles collected from each of several nests on the indicated date. Within a row, means sharing superscripted letters were statistically indistinguishable as determined by a nested ANOVA, followed by Bonferroni multiple contrasts. Statistical data pertain to the "sample date" term of the model.

[a] Reported values were adjusted for significant covariation in carapace length (ANCOVA; $P < 0.05$).

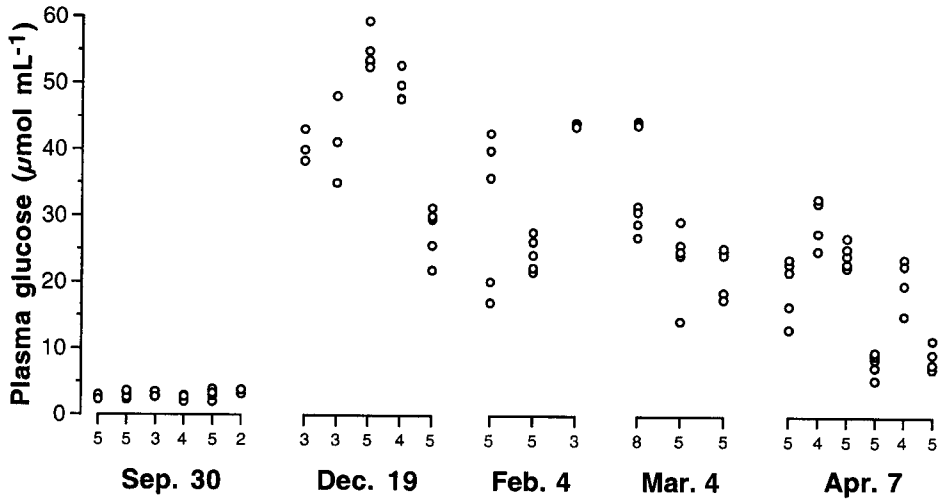

Figure 3. Seasonal, internest, and intranest variation in plasma glucose concentration in hatchling painted turtles collected from three to six nests at a study area in west-central Nebraska (CLNWR) on each of five dates during winter 2000–2001. Each circle represents a value determined for at least one turtle; numerals indicate the number of turtles collected from a given nest for which data are presented.

unusually high lactate levels (10–25 μmol mL^{-1}), turtles did not accumulate large amounts of this metabolite. Furthermore, concentrations were not appreciably higher in 2000–2001 than in 1999–2000, suggesting that plasma lactate level and degree of cold exposure were not strongly associated.

Internest Variation in Somatic Characteristics and Physiology

We found high internest variability in morphometric variables (carapace length, plastron length, fresh body mass, and dry body mass) and in carcass water content (Table 3). Plasma protein concentration varied among CLNWR nests but not among MZMP nests. We found marked internest variation in carcass organic and lipid contents, primarily among the samples collected in late winter (Table 3). However, some physiological attributes (e.g., hematocrit, plasma glycerol), including even seasonally labile ones (e.g., plasma osmolality), showed remarkable uniformity (Table 3). In autumn, plasma glucose levels varied little among turtles inhabiting different nests; however, we found high variability in some winter samples (Table 3), particularly those consisting of hyperglycemic and hyperlactemic turtles that had been subjected to severe chilling (Table 2). For example, mean plasma glucose concentrations determined for each of five CLNWR nests sampled on December 19, 2000, ranged from 27.9 to 55.1 μmol mL^{-1} (Fig. 3). Furthermore, mean plasma lactate concentrations determined for four MZMP nests sampled on February 16, 2000, ranged from 2.0 to 14.9 μmol mL^{-1}.

Correlation analysis revealed some interesting, significant ($P < 0.05$) associations among various somatic and physiological characteristics of overwintering turtles. With respect to water balance, turtles that were heavier (on a dry carcass basis) had lower water contents, and carcass water content was strongly inversely related to both hematocrit and plasma protein concentration. We found a strong, direct correlation between plasma osmolality and plasma glucose level in both winters of study and at each study area, suggesting that this metabolite is an important organic osmolyte. Osmolality also was correlated with lactate and urea, especially among the MZMP turtles. Glucose and urea levels were directly correlated in both CLNWR and MZMP turtles, but only in winter 1999–2000.

When possible, we measured the somatic and physiological characteristics of turtles that exhibited unusual behaviors such as fall emergence and late hatching. On September 30, 2000, we collected three turtles as they emerged from a CLNWR nest that contained the fragmented shells of at least five successfully hatched eggs and one intact, dead egg but no additional hatchlings. With respect to length, mass, hydration state, and metabolite levels, these turtles resembled other hatchlings sampled from within nests on the same day except that their carcass lipid content (141 $\pm$ 7.5 mg g^{-1}; $n = 3$) was ~30% higher. On October 2, 1999, five unpipped eggs were collected from a CLNWR nest, transported to the laboratory, and incubated at ~22°C until they hatched several days later. These neonates appeared normal, although they had exceptionally high hematocrits (35% $\pm$ 2%; $n = 5$). Another of the nests sampled on October 2, 1999, contained eight eggs in various stages of hatching. Neonates emerging from these eggs also had relatively high hematocrits (33% $\pm$ 0.9%; $n = 8$) as well as high concentrations of lipid (159 $\pm$ 4 mg g^{-1}; $n = 8$) but otherwise were

Table 3: Variability in somatic and physiological characteristics of hatchling painted turtles collected from different nests (no. in parentheses) at study areas in west-central Nebraska (CLNWR) and northern Indiana (MZMP) during 2 yr of study

	10/1/99 (5)	1/17/00 (2)	2/27/00 (3)	4/8/00 (5)	
CLNWR, 1999–2000:					
Carapace length	✶✶✶	✶	✶✶✶	✶✶✶	
Plastron length[a]	✶	NS	✶✶✶	✶	
Fresh body mass[a]	✶✶✶	NS	✶✶✶	NS	
Dry body mass[a]	✶✶✶	NS	✶✶✶	✶	
Carcass water content[a]	✶✶✶	NS	✶	✶✶✶	
Plasma osmolality	NS	NS	NS	NS	
Carcass organic content	✶✶✶	✶✶✶	✶✶✶	✶	
Carcass lipid content	✶✶✶	✶✶✶	✶✶	✶✶✶	
Hematocrit	NS	NS	✶	NS	
Plasma glucose	✶	NS	✶✶	NS	
Plasma glycerol	✶	✶	NS	NS	
Plasma lactate	NS	NS	✶	NS	
Plasma urea[a]	NS	✶✶	NS	NS	
Plasma protein	✶	✶	✶✶	✶	
	11/17/99 (4)	2/16/00 (4)	3/23/00 (3)		
MZMP, 1999–2000:					
Carapace length	✶✶✶	✶	✶✶✶		
Plastron length[a]	NS	NS	✶		
Fresh body mass[a]	NS	NS	✶		
Dry body mass[a]	✶✶	✶	✶✶		
Carcass water content[a]	✶✶	✶	✶		
Plasma osmolality	NS	NS	NS		
Carcass organic content	✶✶✶	NS	✶		
Carcass lipid content	✶✶✶	✶	✶✶✶		
Hematocrit	NS	NS	NS		
Plasma glucose	NS	NS	✶		
Plasma glycerol	NS	NS	NS		
Plasma lactate	NS	NS	NS		
Plasma urea[a]	✶	NS	NS		
Plasma protein	NS	NS	✶		
	9/30/00 (6)	12/19/00 (5)	2/4/01 (3)	3/4/01 (3)	4/7/01 (6)
CLNWR, 2000–2001:					
Carapace length	✶✶✶	✶✶✶	✶✶✶	✶✶✶	✶✶✶
Plastron length[a]	✶✶	✶	NS	NS	✶✶
Fresh body mass[a]	✶	✶✶	✶✶✶	NS	✶✶✶
Dry body mass[a]	✶	✶✶✶	✶✶✶	NS	✶✶✶
Carcass water content[a]	✶	✶✶✶	✶	NS	✶✶
Plasma osmolality	✶	✶	NS	NS	✶
Carcass organic content	✶✶✶	NS	NS	✶✶✶	✶✶✶
Carcass lipid content	✶	NS	NS	✶✶✶	✶✶✶
Hematocrit	NS	NS	✶✶✶	✶✶✶	✶✶✶
Plasma glucose	NS	✶✶✶	✶	✶	✶✶✶
Plasma glycerol	✶✶✶	NS	NS	✶	✶
Plasma lactate	NS	NS	NS	✶	NS
Plasma urea[a]	✶✶	NS	✶✶	✶	✶
Plasma protein	✶✶	✶✶	✶	✶✶	✶✶✶
	11/26/00 (5)	1/17/01 (5)	4/2/01 (4)		
MZMP, 2000–2001:					
Carapace length	✶✶	✶✶✶	✶✶✶		
Plastron length[a]	NS	NS	✶✶✶		
Fresh body mass[a]	NS	✶	NS		
Dry body mass[a]	✶✶	✶✶✶	✶✶✶		
Carcass water content[a]	✶✶✶	✶✶✶	✶✶		
Plasma osmolality	NS	NS	NS		
Carcass organic content	✶✶✶	NS	✶		
Carcass lipid content	NS	NS	✶✶✶		
Hematocrit	NS	NS	✶		
Plasma glucose	✶	NS	✶		
Plasma glycerol	NS	NS	✶		
Plasma lactate	NS	NS	✶✶✶		
Plasma urea[a]	NS	NS	✶		
Plasma protein	NS	NS	NS		

Note. Variation among nests sampled on a given date was examined using ANOVA. Significance of differences among means for individual nests are indicated as follows: $P \leq 0.0001$, three asterisks; $P < 0.001$, two asterisks; $P < 0.05$, one asterisk; and $P \geq 0.05$, NS.

[a] ANCOVA was used because the variable significantly ($P < 0.05$) covaried with carapace length.

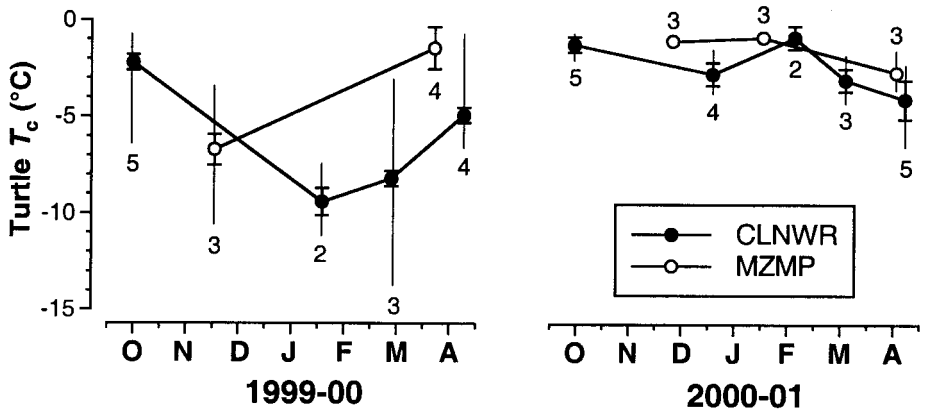

Figure 4. Seasonal variation in resistance to inoculative freezing of hatchling painted turtles collected at study areas in west-central Nebraska (CLNWR) and northern Indiana (MZMP) during 2 yr of study. On each sample date, inoculation resistance was determined for one to five hatchlings representing each nest (number of nests as indicated), and the data for these turtles were averaged to produce a single value. The thin vertical line represents the range in average T_c values determined for individual nests, whereas the least squares mean and SE computed for all turtles collected on the same day are shown by a circle and horizontal bars, respectively.

physiologically indistinguishable from their cohorts; thus, we included these data in the statistical analysis.

Intranest Variation in Somatic Characteristics and Physiology

Within-clutch variability in somatic and physiological attributes was gauged from the associated coefficients of variation (CV), which we computed for each nest that contributed turtles to our physiological studies. Clutch mates varied little ($CV < 0.10$) in morphometrics (carapace and plastron lengths, fresh body mass, dry body mass) and in some somatic and physiological attributes (e.g., carcass organic and water contents, plasma osmolality). However, we found substantial variation ($CV = 0.10$ to 0.15) in carcass lipid content, hematocrit, and plasma protein concentration, and variability was greatest ($CV = 0.15$ to 0.73) in the metabolites (urea, glucose, lactate, and glycerol). Generally, similar patterns of variation among siblings occurred at CLNWR and MZMP, although MZMP clutches showed higher variability in plasma levels of urea and glucose.

Questioning whether siblings might vary in their responses to environmental stress, such as dehydration and cold, we tested the somatic and physiological data for seasonal changes in intranest variability, using ANOVA to compare nests' variances among sample dates. These analyses, performed separately for CLNWR and MZMP in each year of the study, required log transformation of the variances owing to heteroscedasticity among the groups (sample dates) and proportionality of the standard deviations to the means (Zar 1998). We found no seasonal change ($P > 0.05$) in within-clutch variability with respect to morphometrics (carapace and plastron lengths, fresh body mass, dry body mass) and most physiological attributes, although hematocrit and plasma urea concentration became more uniform as the winter progressed. In addition, whereas siblings had similar plasma glucose levels in autumn, we found pronounced variability in glycemia within the clutches sampled during winter. In an extreme case, glucose concentrations in five turtles occupying one nest, excavated on February 4, 2001, ranged from 17.8 to 44.4 μmol mL^{-1} (Fig. 3). In most of the winter samples, however, the greatest difference among siblings was ~15 μmol mL^{-1}.

Resistance to Inoculative Freezing

Hatchlings collected from CLNWR nests in autumn (late September 1999, early October 2000) were highly susceptible to inoculative freezing (Fig. 4). Inoculation resistance improved during winter (Fig. 4), although the increase was more pronounced in 1999–2000 ($F_{3,45} = 47.66$, $P = 0.0001$) than in 2000–2001 ($F_{4,42} = 6.93$, $P = 0.0002$). A seasonal shift in inoculation resistance also occurred in MZMP turtles, in both 1999–2000 ($F_{1,12} = 15.90$, $P = 0.0018$) and 2000–2001 ($F_{2,17} = 13.68$, $P = 0.0003$). Overall, turtles were less susceptible to inoculative freezing in 1999–2000 than in 2000–2001 (Fig. 4).

We examined associations among inoculation resistance and two important characteristics of nest soil, moisture level and activity of constituent INAs, using multiple-regression analyses. We took the average of the T_c values determined for clutch mates to represent the level of inoculation resistance for each nest, regressing these values against corresponding data for soil moisture and INA activity, and the interaction term (soil moisture × soil ice-nucleating activity), which was centered by substituting deviations from the mean values for the original coordinates (Tabachnick and Fidell 2001). We omitted data for CLNWR hatchlings collected in early October 1999 and late

September 2000 because these turtles apparently had not yet developed intrinsic capacities for supercooling or inoculation resistance (Costanzo et al. 2000*b*, 2003; Packard et al. 2001). The regressions indicated that soil moisture was a significant determinant of inoculation resistance at both locales, whereas INA activity level was not (Table 4). The marginally significant interaction term (for MZMP turtles) suggested that INAs acted synergistically with soil moisture to influence inoculation resistance. However, at both locales, soil moisture alone accounted for most of the variation in turtle T_c (Table 4).

Turtles collected from different nests on a given day commonly varied in their resistance to inoculative freezing (Fig. 4). Internest variation was especially great in 1999–2000, both at CLNWR ($F_{10,45} = 18.02$, $P = 0.0001$) and at MZMP ($F_{3,12} = 4.43$, $P = 0.0257$), probably owing to marked heterogeneity in soil moisture (Fig. 2). Notably, of the four CLNWR nests sampled on April 8, 2000, three contained dry soil (i.e., ≤0.042 g water g^{-1} dry soil), and the average T_c values for turtles collected from these nests were ≤−5.5°C. In contrast, the remaining nest contained wet soil (0.155 g water g^{-1} dry soil), and the turtles inhabiting this nest poorly resisted inoculative freezing (average $T_c = -0.8$°C). Similarly, moisture levels in soil collected from MZMP nests on November 17, 1999, were 0.044, 0.064, and 0.223 g water g^{-1} dry soil; corresponding averages of turtle T_c were −10.4°, −6.3°, and −3.3°C, respectively.

Despite being tested in the same substratum, sometimes clutch mates varied markedly in their ability to resist inoculative freezing, as the range in T_c values typically spanned several degrees Celsius. In two extreme cases, T_c values for five siblings from a CLNWR nest ranged from −2.6° to −11.6°C, and T_c values for four siblings collected from one MZMP nest ranged from −1.0° to −9.2°C.

Freeze Tolerance

Whereas few turtles sampled in late September or early October survived our freezing trials, most of the turtles sampled afterward, even in early April, tolerated somatic freezing. This seasonal improvement in freeze tolerance was evident in both years of the study (Fig. 5). Turtles used in these trials were refractory to mechanical stimulation for up to several days following thawing. However, the survivors usually exhibited normal neuromuscular reflexes within 5–6 d.

Inspection of the seven CLNWR nests excavated on February 4, 2001, revealed that many of the turtles were frozen. We intentionally sampled these nests in the midst of an extended period of extreme cold, which, judging from the data recorded in the 13 nests instrumented with temperature loggers, ultimately caused turtles in some nests to remain below 0°C from early November until mid-March and to be exposed immediately before sampling to temperatures ranging from −4.7° to 1.0°C. The soil within the chambers of six of the nests sampled on February 4, 2001, was solidly frozen. Two of the frozen nests held only failed eggs, but the remaining four contained fully formed hatchlings, which, unlike the turtles inside the single unfrozen nest, were rigid, inanimate, and festooned with ice

Table 4: Correlates of inoculation resistance of hatchling painted turtles, as determined by the temperature of crystallization (T_c) of hatchlings cooled in a matrix of frozen nest soil, and characteristics of nest soil collected at study areas in west-central Nebraska (CLNWR) and northern Indiana (MZMP) during 2 yr of study

Independent Variable	sr^2	$b \pm$ SE	t	P
Soil moisture content:				
CLNWR	.195	46.3 ± 19.9	2.38	.028
MZMP	.470	19.2 ± 7.4	2.59	.027
Soil ice-nucleating activity:				
CLNWR	.060	−2.7 ± 1.8	−1.51	.147
MZMP	.002	3.2 ± 1.8	1.76	.108
Soil moisture content × ice-nucleating activity:				
CLNWR	.033	54.1 ± 58.0	.93	.363
MZMP	.174	−36.3 ± 16.4	−2.22	.051

Note. Results of the full-model standard multiple regression analyses are as follows: CLNWR: $F_{3,19} = 2.56$, $P = 0.086$, $r^2 = 0.288$; MZMP: $F_{3,10} = 6.07$, $P = 0.013$, $r^2 = 0.646$. The squared semipartial correlation, sr^2, expresses the unique contribution of the independent variable to the total variance of the dependent variable. The nonstandardized multiple regression coefficient, b, gives the average change in the dependent variable that is associated with a one unit change in the independent variable while holding constant all other independent variables in the model.

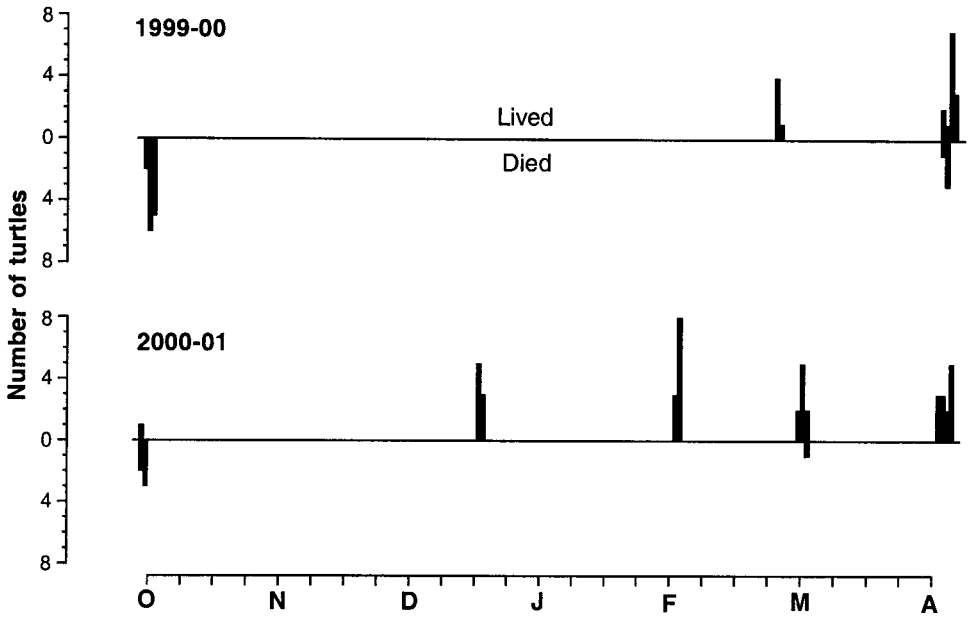

Figure 5. Results of freeze-tolerance trials (72-h exposure to −3°C) conducted with hatchling painted turtles collected at a study area in west-central Nebraska (CLNWR) during 2 yr of study. Each bar shows the outcome (number of turtles alive and dead) of a freeze-tolerance trial involving up to eight turtles representing each nest.

crystals and small clumps of frozen soil. We held all of these turtles at 4°C for 48 h before assessing their viability status and then used the live animals in physiological assays, tests of inoculative freezing, or freeze-tolerance trials. In two of the frozen nests, every hatchling was dead; in another, every hatchling was alive. Three of the 10 hatchlings occupying the remaining frozen nest also had survived somatic freezing under field conditions. Physiological characteristics of the surviving turtles were similar to those of CLNWR turtles sampled 6 wk earlier, when nest temperatures had recently rewarmed to ~0°C (Table 2).

Discussion

Microenvironmental Conditions within the Hibernaculum during Winter

Our study provided a unique opportunity to examine the physiological ecology of hatchling *Chrysemys picta* under contrasting environmental conditions. Winters at CLNWR usually are cold and dry, whereas those at MZMP tend to be more moderate and wetter; soils at CLNWR are friable, porous sands and loamy sands (Costanzo et al. 1995), whereas soils at MZMP are clayey loams (Costanzo et al. 2001*c*). These distinctions were apparent in our 2-yr study. In addition, we were fortunate that winter severity varied annually, providing a rare glimpse at the phenotypic plasticity shown by hatchlings in response to environmental stresses.

Hatchling *C. picta* overwintering inside their natal nests were exposed to cold, although the intensity and duration of the exposure differed markedly between study areas. In northern regions where snow cover is scarce, frost can penetrate rapidly and to great depths in friable soils (Sharratt et al. 1999), possibly exposing hatchlings to subfreezing temperatures. Our data for Nebraska *C. picta*, like those reported previously (Costanzo et al. 1995; Packard 1997), indicate that most such episodes are brief, with hatchlings reaching temperatures only a few degrees below 0°C before rewarming. However, extended chilling episodes do occur, at least during severe winters (e.g., 2000–2001), and these may expose turtles to perilously low temperatures, sometimes with lethal consequences (Table 1). In contrast, instances in which frost penetrates turtle nests are relatively uncommon in more temperate areas, especially when snowfall is abundant (Breitenbach et al. 1984; Nagle et al. 2000). Accordingly, in these areas the threat of mortality due to freezing or profound supercooling (Packard and Packard 1999; Hartley et al. 2000) is relatively low. Owing in part to the insulating effect of snow cover, relatively mild temperatures prevailed in MZMP nests, despite the fact that they were shallower than CLNWR nests. In locales where winter mortality routinely is slight, hibernation in shallow nests may be advantageous if hatchlings responding earlier to rising spring temperatures can sooner leave the nest and begin feeding.

Edaphics significantly affect the winter survival of hatchling *C. picta* because, in addition to their influence on water economy, they play a major role in determining the likelihood of

surviving episodic exposures to subfreezing temperatures. Among soil-dwelling animals, susceptibility to inoculative freezing depends, in part, on various characteristics of the soil (e.g., moisture content, water potential, particle size, porosity) that determine ice abundance and distribution within the microenvironment (Lundheim and Zachariassen 1993; Costanzo et al. 1997, 1998). For example, by adsorbing water, clays and organic matter may attenuate and/or limit the formation of ice within soil pores, thereby limiting an animal's exposure to ice and INAs. For this reason, all else being equal, risk of freezing is greater at CLNWR than at MZMP even though MZMP soils remain wetter throughout the winter (Costanzo et al. 2001*c*). At some locales, *C. picta* prefer clayey soil over sand as a nesting substratum (Legler 1954). By nesting in clayey soil, where locally available, a turtle improves not only her offspring's hatching success (Ratterman and Ackerman 1989) but also their ability to survive at very low temperatures (Costanzo et al. 1998). Interestingly, DePari (1996) found that hatchlings occupying sandy nests were more likely to emerge in fall than turtles inhabiting nests constructed in organic or loamy soils.

The hydration state of nesting soil was linked to regional precipitation patterns as well as its texture and moisture-retention properties. Soils were wettest in midwinter, when nest temperatures were lowest. Maximal activity of organic INAs in nesting soil also occurred in midwinter (see Costanzo et al. 2000*a*), which is suggestive of their association with certain specialized microbes exhibiting thermal sensitivity in the expression of ice-nucleation proteins (Rogers et al. 1987; Gurian-Sherman and Lindow 1995). Because moisture may potentiate the efficacy of these INAs (Table 4; Costanzo et al. 2000*a*), hatchlings are especially susceptible to inoculative freezing at this time.

Levels of soil moisture and INA activity at the MZMP permanent stations were comparable to those found in the chambers of turtle nests, suggesting that females do not discriminate among potential nesting sites on the basis of INA abundance or potency. Indeed, our data demonstrate that nesting habitat is strikingly heterogeneous with respect to thermal and moisture regimes as well as INA activity. Variation in these factors bear strongly on hatchling water balance, energy use, and susceptibility to inoculative freezing, all of which may affect winter survival. Given the patchiness of relatively "safe" overwintering sites on the nesting landscape, distributing ones' eggs among several nests each year may be an effective hedge against complete loss of the annual reproductive effort (Christiansen and Moll 1973). In principle, producing multiple clutches should be of greatest benefit to turtles using habitats in which the risk of hatchling mortality is high. Concordantly, estimated average annual clutch frequency in the CLNWR population is 2.5-fold higher than in the MZMP population (Table 5). At CLNWR, selection seems to be favoring the production of a second or even third clutch, even though these later clutches are provisioned with relatively less energy, are smaller, and are oviposited perilously late in the nesting season (Iverson and Smith 1993). By this reasoning, one might expect clutch frequency to increase with latitude. However, clutch frequency generally is lower in northern populations, perhaps owing to constraints imposed by low energy availability and/or brevity of the season for nesting and egg incubation (Moll 1973; Iverson 1992; Iverson and Smith 1993).

Table 5: Comparison of life-history traits of painted turtles indigenous to study areas in west-central Nebraska (CLNWR) and northern Indiana (MZMP)

	CLNWR		MZMP	
Trait	Mean	*n*	Mean	*n*
Neonatal hatchling:				
Carapace length (mm)	24.0	111	24.1	50
Body mass (g)	4.42	111	4.39	50
Mature female:				
Carapace length (mm)	181.1	127	151.6	155
Body mass (g)	848	127	410	155
Annual clutch frequency (no. clutches per female)	2.78[a]	221	1.13[b]	119
Clutch size (no. eggs per clutch)	13.9[a]	41	6.8[b]	76

Note. Values for hatchlings are composite averages computed for turtles in the initial (autumn) sample collected in each year of this project. Morphometric values for mature females, sampled shortly after oviposition, are composite averages of data collected over several nesting seasons (CLNWR: 2001, 2002; MZMP: 1999–2002).

[a] From Iverson and Smith (1973).

[b] From P. J. Baker and P. E. Meyer (unpublished data).

Hatching and Emergence Anomalies

At both study areas, *C. picta* usually overwintered within the nest chamber, emerging in early spring when soil temperature began to rise. This behavior may benefit turtles by minimizing mortality until such time when resource availability offsets the risk of predation (Gibbons and Nelson 1978). Nevertheless, at each study area, hatchlings from a few nests emerged in fall and apparently moved to water for overwintering. Frequency of fall emergence in northern populations of *C. picta* ranges from low (St. Clair and Gregory 1990) to moderate (Ernst 1971; DePari 1996). The stimulus inducing certain nests to emerge in autumn is unknown, although at some locales soil friability may be a factor (DePari 1996). In a study of *C. picta* in New Jersey, DePari (1996) found that fall-emerging hatchlings had shorter plastrons but weighed the same and had similar amounts of residual yolk as turtles that overwintered inside nests. Accordingly, we found little difference in the physiology of hatchlings emerging in early October, save for unusually high lipid contents. Large lipid reserves, as well as high hematocrits,

were also found in full-term unhatched turtles examined on the same date, raising the possibility that the turtles we found abroad had emerged shortly after hatching.

It is commonly assumed that in order to overwinter successfully, hatchlings must be both developmentally complete and free from the eggshell. However, one of the nests we examined in January contained two intact eggs that held viable hatchlings. We cannot be certain that these eggs ultimately would have hatched in situ; however, both of them hatched (unaided) in the laboratory. This observation raises an interesting question about the cost/benefit trade-offs associated with delayed hatching. Would the deleterious effects of diminished gas exchange be offset by greater thermal insulation and diminished risk of inoculative freezing? Little has been reported about the incidence of delayed hatching, perhaps because few investigators have examined the contents of turtle nests during winter, but our experience suggests it occurs at least occasionally. For example, during our visit to CLNWR on March 31, 2003, we excavated 36 nests that held turtles, eight of which contained up to 11 unhatched live turtles.

Physiological Responses to a Variable Winter Microenvironment

Recording nest temperatures and measuring soil moisture levels permitted us to associate seasonal changes in physiology with changes in the thermal and hydric characteristics of the nest microenvironment. Turtles from both study areas showed similar plasticity in their physiological responses to environmental stress. Generally, the physiological state of turtles studied during the mild, dry winter of 1999–2000 reflected water stress, whereas that of turtles examined during the colder, relatively wet winter of 2000–2001 reflected hypothermic stress.

Dehydration may be a significant mortality factor among terrestrially overwintering reptiles (Gregory 1982). Hibernating within the frost zone can subject turtles to chronic water stress because the vapor pressure of ice is below that of unfrozen body fluids (Forge and MacGuidwin 1992; Lundheim and Zachariassen 1993). Morphological and physiological mechanisms of water conservation thus may be paramount to the winter survival of terrestrially hibernating turtles (Costanzo et al. 2001*b*) as well as other ectotherms (Ring and Danks 1994; Danks 2000; Williams et al. 2002).

Our results suggest that the hydration state of overwintering hatchlings is influenced by local abundance of precipitation and soil moisture. During the unusually dry winter of 1999–2000, turtles tended to lose water and increase plasma levels of urea and other solutes, indicating that they were in water deficit. In contrast, turtles remained in positive water balance throughout the wetter winter of 2000–2001, showing no urea accumulation and an apparent net gain in water content. In the laboratory, hatchling *C. picta* overwintering under a favorable moisture regimen also gained water (Costanzo et al. 2000*b*), which presumably was endogenously produced because hatchlings cannot absorb moisture from their environment (Costanzo et al. 2001*b*) and it is unlikely that they imbibe water while inside the nest. Some tortoises reportedly obtain water from a humid atmosphere (Wilson et al. 2001), although such "vapor influx" has not been measured directly. We cannot rule out the possibility that hatchling *C. picta* can obtain at least some water from the nest environment; however, the apparent net gain realized under favorable winter conditions may reflect adaptations that limit evaporative water loss (Costanzo et al. 2001*b*) coupled with production of metabolic water via β-oxidation of fatty acids. Perhaps the large lipid and yolk reserves in hatchling *C. picta*, as compared to those in aquatic hibernators (Congdon and Gibbons 1990; Costanzo et al. 2000*b*), are especially beneficial in dry years.

We do not know the extent to which hatchling *C. picta* dehydrate during winter, nor whether water loss is an important physiological stress. In order to address this question, we conducted a simple experiment based on the premise that dehydrated hatchlings given access to standing water will imbibe only the volume required to restore water balance (Costanzo et al. 2001*b*). When 10 hatchlings were collected from a single CLNWR nest on April 8, 2000, and held overnight in shallow water (~20°C), they gained 11.4% $\pm$ 1.1% of their initial body mass. On repeating the experiment at the end of the subsequent, wetter winter (April 7, 2001), body mass changed only slightly (range of means: $-4.5\% \pm 0.4\%$ to $0.1\% \pm 0.4\%$; 4–9 turtles per nest; $n = 8$ nests), indicating that these turtles did not drink, perhaps because they were fully hydrated. Our final experiment, conducted on April 6, 2002, following a very dry winter, further suggested that hatchling *C. picta* may dehydrate substantially, as these turtles ($n = 6$–9 turtles per nest; $n = 8$ nests) gained from $1.6\% \pm 1.6\%$ to $10.2\% \pm 1.2\%$ of their initial body mass on immersion in water. Because availability of environmental moisture during embryonic development influences the water reserve in hatchlings (Ratterman and Ackerman 1989; Packard 1991), dehydration stress may be exceptionally great during a cold, dry winter that follows a droughty summer. Recent work suggests that water balance may strongly influence the fitness of hatchlings after emergence from the nest (Finkler 1999; Tucker and Paukstis 1999; Finkler et al. 2000).

Hematocrit varied little during winter, suggesting that hatchlings regulated plasma volume in the face of a dynamic water balance. Defense of the plasma volume while under desiccation stress may enable turtles to maintain proper blood viscosity and performance of the circulatory system (Peterson and Stone 2000).

In both years of the study and at each study area, the decrease in dry body mass over the course of a 6-mo winter was slight, if even perceptible, indicating that the energy requirement during hibernation was minimal. Indeed, hatchlings overwintering in the laboratory apparently gain weight, owing (at least in part) to the biosynthesis of water (Filoramo and Janzen 1999; Costanzo et al. 2000*b*). However, we found that the seasonal

decrease in lipids and other energy-yielding substrates was greater in 1999–2000, probably owing to a higher metabolic demand imposed by the relatively warm environment. Similarly, MZMP turtles expended more energy, perhaps because their nests were warmer than CLNWR nests (Fig. 1).

Lipids are an important energy source in many hibernating reptiles (Derickson 1976; Gregory 1982). During winter 1999–2000, MZMP turtles consumed up to one-quarter of their total lipids, but the resulting energy (~1.22 kJ) contributed less than one-half of the total energy (2.70 kJ) obtained from catabolism of both lipid and nonlipid substrates in the average-size turtle. Furthermore, turtles used lipid reserves sparingly, if at all, during the subsequent colder winter. This outcome may reflect a very low level of basal metabolism and/or an inability to mobilize lipid at low body temperature, especially because cells become hypoxic during somatic freezing (Storey 1990) and supercooling (Hartley et al. 2000; Costanzo et al. 2001*a*). Reptiles that spare their lipid reserves during hibernation have a rich energy source to fuel postemergence activities (Parker and Brown 1980; Gregory 1982; Costanzo 1985).

In neonatal turtles, plasma levels of urea, the primary catabolic product of protein metabolism, are strongly influenced by the environmental moisture regime during embryonic development (Packard and Packard 1989). Comparing our results for 1999–2000 and 2000–2001 showed that urea levels in overwintering hatchlings also depend on hydration state. Like other vertebrate ectotherms, turtles accumulate urea during water stress (Dantzler and Schmidt-Nielsen 1966; Baze and Horne 1970). In high concentrations, urea may depress metabolism (Yancey et al. 1982), which in principle would benefit overwintering hatchlings (Costanzo et al. 2000*b*).

The seasonal increase in urea levels suggests that hatchlings actively metabolize protein during winter. Previous study has shown that, during simulated hibernation, hatchling *C. picta* (but not *Chelydra serpentina*) maintain a rich pool of amino acids (Costanzo et al. 2000*b*). These compounds may serve as "compatible osmolytes," protecting cells against excessive dehydration, and some, such as proline, may protect against both freezing and desiccation stresses (Takagi et al. 2000). Additionally, amino acids can be metabolized by hypoxic tissues (Van Waarde 1988), with the resulting carbon skeletons converted to the cryoprotectant glucose via gluconeogenesis. This pathway may also be active during desiccation, as evidenced by the strong correlation between urea and glucose levels in our 1999–2000 turtles.

The transient elevation in plasma protein in our winter turtles may reflect a history of tissue freezing. Protein levels are unaffected by cold acclimation (Costanzo et al. 2000*b*) but increase slightly in response to somatic freezing (Storey et al. 1991). Relative to the major inorganic solutes and various organic compounds, proteins are particularly efficient at retarding ice-crystal growth (Lusena 1955). This effect may be beneficial because slow freezing is requisite for freeze tolerance (Storey and Storey 1992), although it is uncertain whether the relatively small increase in protein concentration would promote freezing survival via this effect.

In high concentrations, glycerol promotes supercooling in freeze-avoiding insects and limits freezing-induced cell dehydration in freeze-tolerant insects (Zachariassen 1985; Storey 1990). Glycerol also plays a cryoprotective role in vertebrate ectotherms (Berman et al. 1984; Layne and Jones 2001), albeit much less commonly. In our study, overwintering hatchlings maintained low levels of glycerol. Our finding concurs with laboratory studies reporting that hatchlings do not accumulate high levels of this polyol during cold acclimation (Costanzo et al. 2000*b*), somatic freezing (Storey et al. 1988; Churchill and Storey 1992*a*), or supercooling (Costanzo et al. 2001*a*).

In contrast, plasma glucose levels increased markedly during early winter. Concentrations exceeding the basal level (3–5 μmol mL^{-1}) were first observed after frost reached the nests, suggesting that the trigger for glucose mobilization is acute chilling. This assertion is supported by laboratory studies demonstrating that hyperglycemia is induced by supercooling (Costanzo et al. 2001*a*) and by somatic freezing (Churchill and Storey 1991) but not by gradual cold acclimation (Costanzo et al. 2000*b*). In addition, our finding that glucose levels were higher in winter 2000–2001 and in the CLNWR turtles suggests that degree of chilling modulates the glycemic response. We found no evidence that *Chrysemys picta bellii* and *Chrysemys picta marginata* differ in their glycemic responses to hypothermia, as was suggested by Churchill and Storey (1991).

Supercooled turtles may benefit from high levels of glucose, which may act both as an osmotic agent and as a metabolic substratum (Costanzo et al. 2001*a*). However, as a cryoprotectant, its better-known role is to limit ice content and the attendant cell shrinkage in frozen tissues (Storey and Storey 1992). Some anurans can achieve blood glucose levels of 250 μmol mL^{-1} during somatic freezing (Storey and Storey 1986). In contrast, maximal concentrations only one-tenth as great are attained by freeze-tolerant squamates (Costanzo et al. 1988; Voituron et al. 2002) and turtles (Storey et al. 1988; Churchill and Storey 1992*b*; Costanzo et al. 1993; Hemmings and Storey 2000), calling into question the role of glucose as a colligative agent among reptiles. However, in our study, turtles subjected to natural chilling episodes became markedly hyperglycemic, with glucose levels in some individuals reaching 60 μmol mL^{-1}, about twofold higher than those reported previously, suggesting that field acclimatization may be critical to the expression of a maximal cryoprotectant response.

In laboratory trials, tissue lactate levels in hatchling *C. picta* increased markedly (up to 15–25 μmol g^{-1}) during supercooling (Hartley et al. 2000; Costanzo et al. 2001*a*) and somatic freezing (Storey et al. 1988; Churchill and Storey 1992*b*; Hemmings and Storey 2000), probably owing to diminished function of the oxygen delivery system. Some workers (Churchill and Storey 1991; Storey and Storey 1992) speculate that lactate

rather than glucose is the primary colligative cryoprotectant used by hatchling *C. picta*. Using lactate has the advantage of producing twice as many moles of solute from the same mole of hexose phosphate. On the other hand, the associated lactacidosis may adversely impact organismal fitness. Adult *C. picta* are well known for their capacity to tolerate high lactate loads; however, hatchlings lack the extensive mineralization of the shell critical to the buffering system (G. R. Ultsch and S. A. Reese, unpublished data). Furthermore, lactate buffering in deeply supercooled hatchlings may be hampered by hypoxic ischemia (Hartley et al. 2000). Ultimately, excessive accumulation of lactate may be associated with mortality in chronically supercooled hatchlings (Packard and Packard 1999; Hartley et al. 2000). Moreover, noting that hatchlings recovering from supercooling exhibit impaired behavioral functions, Hartley et al. (2000) surmised that elevated lactate may delay or hamper emergence from the nest. In our study, plasma lactate levels increased about threefold during winter, probably when turtles froze or became supercooled, but even the maximal levels attained probably were insufficient either to provide significant cryoprotection or to impair physiological function.

Mechanisms of Cold-Hardiness: Supercooling, Inoculation Resistance, and Freeze Tolerance

Whereas hatchling *C. picta* can survive in the supercooled state to −12°C (Paukstis et al. 1989; Packard and Packard 1993, 1999; Costanzo et al. 1999), they can tolerate somatic freezing only if their body temperature does not descend below −3° to −4°C (Storey et al. 1988; Churchill and Storey 1992*b*; Costanzo et al. 1995; Packard et al. 1999*b*). Clearly, supercooling offers protection over a broader thermal range; however, using this strategy requires hatchlings to avoid spontaneous ice nucleation and inoculation by external ice and INAs, since even freeze-tolerant animals succumb to the crystallization of deeply supercooled tissues (Storey and Storey 1988). Freezing survival is promoted when ice nucleation occurs at a relatively high temperature because the cells and tissues can better cope with the attendant osmotic stress.

With the approach of winter, many temperate ectotherms undergo marked physiological changes that enable them to survive the ensuing cold. Development of supercooling capacity often is associated with elimination of ingested food and other INAs, accumulation of low molecular weight cryoprotectants, and production of antifreeze (= thermal hysteresis) proteins (Block and Zettel 1980; Sømme 1982; Zachariassen 1985; Bale et al. 1989; Duman et al. 1995). Because turtles hatching in natural nests invariably are contaminated with INAs (Costanzo et al. 2000*a*, 2003), we were unable to investigate the dynamics of intrinsic supercooling capacity. However, laboratory studies involving turtles reared in an INA-free environment have confirmed that supercooling increases seasonally (Costanzo et al. 2000*b*; Packard et al. 2001). Recent work suggests that the enhancement stems from gut evacuation of both ingested and endogenously produced INAs (Packard et al. 2001; Costanzo et al. 2000*b*, 2003) but does not involve antifreeze agents or any of the common cryoprotectants (Costanzo et al. 2000*b*).

Some authors avow that in order to survive a cold winter inside the nest, hatchling *C. picta* need invoke only their capacity to supercool (Packard and Packard 2001*b*). However, this argument derives from study of turtles reared on artificial substrata and tested under ecologically irrelevant conditions, such that the results tend to overestimate inoculation resistance (Costanzo et al. 2000*a*). To the contrary, a growing body of literature has established that, under certain environmental conditions, hatchling *C. picta* are highly susceptible to inoculation by ice and INAs, which may include dust and various soil particulates, complexes of inorganic and organic entities formed by the decay of organic materials, and certain microorganisms, all of which may occur in the nest environment (Costanzo et al. 2000*a*). In addition to temperature, the probability of inoculation depends on the type, potency, and abundance of INAs and their degree of intimacy with the body surface (Lee and Costanzo 1998), as well as the efficacy of any anatomical barrier (Willard et al. 2000; Costanzo et al. 2001*b*). Characteristics of nesting soil, such as water potential, texture, and porosity, may strongly influence these factors (Packard and Packard 1997; Costanzo et al. 1998, 2000*a*, 2001*c*). Moisture content is a particularly important variable but must be considered in relation to the moisture-binding capacity of the soil (Costanzo et al. 1998). We designed our tests of inoculative resistance to assess the response of turtles under natural, seasonally dynamic conditions within their nests.

The variation in inoculation resistance between *C. p. bellii* and *C. p. marginata* suggested by our data probably reflected different characteristics of CLNWR and MZMP soils rather than populational differences per se. With moisture level held constant, susceptibility to inoculative freezing is higher for both subspecies when turtles are exposed to the sandy soil indigenous to CLNWR as compared to the MZMP clayey loam (Costanzo et al. 2001*c*). We suspect that soil texture contributes significantly to variation in winter survival on a regional scale.

Laboratory study of hatchling *C. picta* has shown that inoculation resistance, like innate supercooling capacity, improves with cold acclimation, seemingly to prepare turtles for hibernation (Costanzo et al. 2000*b*). This pattern appears in our results for the CLNWR turtles examined in winter 1999–2000; hatchlings sampled in early autumn were highly susceptible to inoculative freezing (Fig. 4) despite the presence of dry soil in their nests (Fig. 2), whereas, during winter, resistance generally improved in the face of increasing levels of environmental moisture and INA activity.

The morphological and/or physiological adjustments underlying the seasonal development of inoculation resistance in this species are as yet unknown. These changes are critical to the survival of turtles overwintering in nests, at least in regions

where temperatures may fall substantially below the equilibrium freezing point of tissues. Nevertheless, the ability of even winter-acclimatized hatchlings to resist inoculative freezing is limited. For example, turtles were prone to freezing throughout winter 2000–2001, probably because nest soil remained damp over much of the study period. Assuming that freezing is a primary cause of death, winter mortality ought to be greater in winters that are both wet and cold, and this contention is supported by the results of our study.

Hatchling *C. picta* can survive periods of somatic freezing lasting up to 11 d and can tolerate the freezing of up to 53% of their body water (Storey et al. 1988; Churchill and Storey 1992*b*). Citing cases in which nest temperature cools below the critical thermal minimum, some authors have questioned the ecological relevance of freeze tolerance in this species (Packard and Packard 2001*b*). However, our recordings of nest temperature suggest that hatchlings can survive winter routinely at MZMP (and similar temperate locales) and during relatively mild winters (e.g., 1999–2000) at CLNWR, wholly by virtue of their freeze tolerance. Freeze tolerance probably is of greatest importance to the survival of hatchling *C. picta* during wet winters because the high likelihood of being inoculated by environmental ice or INAs precludes supercooling.

Our study not only provides the first direct evidence for freezing survival of hatchlings in nature but it also conclusively demonstrates that freeze tolerance, like supercooling capacity and inoculation resistance, develops during acclimatization to winter conditions. Churchill and Storey (1992*b*) reported that hatchling *C. p. marginata*, collected from their nests at the end of winter, were substantially more freeze tolerant than a November cohort, suggesting that cold-hardening is required for the full expression of freeze tolerance. The physiological basis underpinning the transition is unknown but may involve shifts in plasma membrane composition, structure, and function that render this sensitive structure more tolerant to freezing-induced stresses (Orvar et al. 2000). We caution that laboratory studies may underestimate the capacity for freeze tolerance in hatchling turtles if they do not use field-acclimatized animals, and furthermore, if they do not allow adequate time for recovery of neurobehavioral functions before assessing survival.

Inter- and Intranest Variation in Winter Physiological Ecology

Our investigation of hatchling *C. picta* spanning two consecutive but contrasting winters, in distinct habitats, illuminated the value of phenotypic plasticity in physiological response to survival in a nest environment that is often harsh and variable in time and space. Also instructive was the pronounced inter- and intranest variation in traits influencing winter survival. Commonly, we found marked variability in turtles sampled on the same day, even though the majority of nests were separated by no more than several meters. Such variation may reflect adaptive phenotypic response to spatial heterogeneity in edaphics and other abiotic factors (Stearns 1989; Gotthard and Nylin 1995); it may also result from a maternal or "clutch" effect, the combined influence of genetic constitution and maternal investment on an individual's response to environmental challenge (Steyermark and Spotila 2000, 2001; Packard and Packard 2001*a*). We also found substantial phenotypic variation among individuals of at least the same maternal parentage occupying the same nest. Interindividual variation in physiological performance may have important ecological and evolutionary implications (Arnold 1983; Spicer and Gaston 1999).

Maternal choice of nesting site is an important, albeit little studied, factor shaping the life-history evolution of oviparous species that lack parental care (Resetarits 1996). Because the landscape on which freshwater turtles nest is a complex, temporal mosaic of varying thermal and hydric regimes, choice of oviposition site can have a strong impact on embryonic development and nest success (Ratterman and Ackerman 1989; Cagle et al. 1993; Wilson 1998; Kolbe and Janzen 2002) as well as the phenotypic characteristics of hatchlings, such as gender, body size, and body composition (Schwarzkopf and Brooks 1987; Janzen 1994; DePari 1996; Packard et al. 1999*a*, 2000; Weisrock and Janzen 1999; Packard and Packard 2000, 2001*a*). A spate of recent studies has addressed the impact of environmental heterogeneity on the postemergence performance or fitness of hatchlings (Miller et al. 1987; O'Steen 1998; Finkler 1999; Rhen and Lang 1999; Tucker and Paukstis 1999; Finkler et al. 2000; Kolbe and Janzen 2001; Steyermark and Spotila 2001; Janzen and Morjan 2002); however, the influence of nesting site location on the winter ecology of turtles has received relatively little study (Breitenbach et al. 1984; Weisrock and Janzen 1999; Nagle et al. 2000).

Internest variation in the physiology of overwintering hatchlings likely reflects spatial heterogeneity in environmental conditions at the nesting site. For example, differential exposure to severe cold may account for variation in glucose and lactate levels, as found in our winter samples. In addition, variation in prevailing temperature and hydric regimens, perhaps due to slope and aspect, may account for the observed variation in water balance (carcass water content) and energy level (organic and lipid content), although maternal effects (e.g., differential provisioning of the eggs) may also be involved.

Studies of overwintering in hatchling *C. picta* commonly show pronounced internest variability in the intensity and duration of subfreezing cold (Breitenbach et al. 1984; Packard et al. 1989, 1997; Paukstis et al. 1989; Packard 1997; Nagle et al. 2000). Because these measures do not always correlate with winter survival (Packard et al. 1989), viability must be influenced by additional, possibly interactive, environmental factors. For example, Packard et al. (1997) determined the probability of winter survival for hatchling *C. picta*, in north-central Nebraska, as a function of minimum nest temperature, reporting the LT_{50} to be approximately -10°C. However, survival in individual nests incurring similar thermal minima actually ranged

from 0% to 88%. Inspection of these data reveals that the nests constructed in loamy sand fared much better than the nests constructed in fine sand, corroborating laboratory findings (Costanzo et al. 1998) that soil texture influences winter survival of hatchling turtles. The degree to which conditions promoting winter survival factor into maternal choice of oviposition site is a subject worthy of study.

Eggs or hatchlings occupying a three-dimensional nest chamber may be exposed to a range of thermal (Ratterman and Ackerman 1989; Tucker 1999) and hydric (Legler 1954; Hotaling et al. 1985) conditions, such that position within clutch may modulate expression of phenotypic traits that ultimately affect fitness (Packard and Packard 2001*a*). In our study, marked intranest variation in several physiological characteristics (e.g., cryoprotectant levels, lipid reserves, etc.) may reflect such heterogeneity, although potential differences in hatchling "quality" may also play a role. In addition, clutch mates sometimes varied in their ability to resist inoculative freezing and to survive somatic freezing, despite being evaluated under identical conditions. Factors underpinning phenotypic variation in these attributes may relate to an individual's physiological status and/or quality of the anatomical barrier to the inward propagation of environmental ice or INAs. In particular, inoculation resistance may be relatively poor in hatchlings that have not deposited sufficient lipid in the integument (Willard et al. 2000).

Influence of the Winter Environment on Life-History Traits

Some workers (Costanzo et al. 2000*a*, 2001*c*; Nagle et al. 2000) have questioned whether the arrangement and location of the hatchlings within the nest chamber may influence the risk for freezing mortality. Hatchling *C. picta* and other terrestrially hibernating species (Tucker 1997; Andreas and Paul 1998) reportedly cluster above eggshell fragments, with their heads near the top of the nest chamber (Breitenbach et al. 1984; Packard et al. 1989; St. Clair and Gregory 1990; DePari 1996; Nagle et al. 2000). Turtles near the periphery are predisposed to freezing because they are most intimate with soil (Hotaling et al. 1985) and, consequently, also with ice and INAs, whereas such hazards are better avoided by turtles nearer the nest's center. This scenario assumes that the nest chamber remains well formed and relatively free of infiltrating soil, but this is not always the case. Regional variation in soil texture and friability likely distinguishes nests that retain their form (Breitenbach et al. 1984; DePari 1996) from those that become infiltrated (Hartweg 1944; Packard et al. 1989). Nevertheless, differential freezing risk may explain the rather common instances of mixed survival of turtles inhabiting the same nest (Packard et al. 1989, 1997; Lindeman 1991; DePari 1996; Weisrock and Janzen 1999; Nagle et al. 2000). Furthermore, because the primary threat of mortality is from contact with environmental ice and INAs, and because relatively protected internal positions can exist only in nests containing many hatchlings, in principle a larger clutch should have a survival advantage in cold winters. Our finding that each of the CLNWR nests sampled on April 7, 2001, having 100% winter survival contained at least 9 hatchlings prompted us to perform post hoc analyses that seem to support this contention: survival in nests containing >9 hatchlings (mean $\pm$ SE = 77% $\pm$ 7%; $n = 27$) was higher (Student's t-test: $P = 0.027$) than survival (45% $\pm$ 15%; $n = 9$) in nests containing $\leq$9 hatchlings. Also, incidence of complete winterkill was lower in nests containing >9 hatchlings (11.1% vs. 44.4%; Fisher's exact, $P = 0.049$), and, overall, more hatchlings emerged in spring from these nests (10 vs. 3 hatchlings, on average).

This concept not only argues for the likelihood of strong directional selection for large clutches in turtles living at high latitudes and in cold habitats but also poses an intriguing challenge to classical optimizing selection models (Lack 1954; Sinervo and Svensson 1998; Sinervo 1999; Ricklefs 2000). Northern populations of *C. picta* (and other species) produce relatively large clutches, purportedly to offset a higher mortality associated with life in an extreme environment (Tinkle 1961; Gemmell 1970; Moll 1973; Iverson and Smith 1993; Iverson et al. 1993). This "protected siblings" hypothesis offers a novel mechanism on which such selection might operate with respect to terrestrially hibernating hatchlings. Note, however, that it does not preclude the possibility that large clutches also are beneficial, albeit through some other mechanism, in the more northern populations of species whose hatchlings hibernate underwater (e.g., *C. serpentina*, *Apalone spinifera*). Because CLNWR females produce twice as many eggs per clutch as MZMP females (Table 5) even though they live at the same latitude, producing large clutches may be an adaptive response to severe environmental conditions rather than increasing latitude per se.

It is commonly understood that body size influences many aspects of an organism's physiology and ecology, although little is known about the relationship between body size and winter survival among ectotherms in general and among turtles in particular (Bodie and Semlitsch 2000). Students of allometry have long advocated that being large confers significant survival advantage (Lack 1954; Sinervo et al. 1992; Chown and Gaston 1997). Regarding hatchling turtles, many studies (though certainly not all; see Brooks et al. 1991; Bobyn and Brooks 1994; Congdon et al. 1999) support the "bigger is better" tenet with respect to postemergence survival, growth, and other measures of organismal performance (e.g., Haskell et al. 1996; Janzen et al. 2000; Tucker 2000*a*). Contrary to this paradigm, ice nucleation theory predicts that small size should favor survival in species that rely on supercooling to survive extreme chilling. Ice nucleation occurs once sufficient water molecules have organized to form an ice embryo (Rasmussen and MacKenzie 1973), and, because the probability of embryo formation increases with fluid volume (Vali 1995), small animals tend to

supercool more extensively than large ones (Costanzo and Lee 1995; Lee and Costanzo 1998; Wharton 2002). In addition, by virtue of their limited body surface, small animals may be less susceptible to inoculation by environmental ice and INAs. Among hatchling turtles, inoculative freezing is mediated at least in part by the integument (Willard et al. 2000); accordingly, freezing resistance is inversely related to the area of the exposed skin surface (Costanzo et al. 2001*b*).

Presently, we lack sufficient field data to rigorously test the hypothesis that small hatchlings have a winter survival advantage over large ones. Nevertheless, northern turtles potentially can lay eggs larger than those actually produced (assuming that pelvic aperture is a limiting factor; Iverson and Smith 1993; Tucker 2000*b*), and if winter severity imposes a strong selective pressure favoring production of hatchlings of smaller than maximal size, one might predict that neonates at CLNWR would be smaller as compared to those at MZMP. We found no differences in morphometrics between these populations (Tables 2, 5). However, considering that egg (and hatchling) size tends to vary directly with maternal body size in *C. picta* (Iverson and Smith 1993) and that the average CLNWR female weighs twice as much as the average MZMP female (Table 5), hatchlings produced in the more severe habitat are in fact relatively small.

Among populations of *C. picta*, annual reproductive output seems to remain constant relative to body size (Iverson and Smith 1993) and is partitioned within the clutch via a tradeoff between offspring number and offspring size (Iverson 1992). Therefore, a female producing relatively small hatchlings also produces many hatchlings, and if both small body size and large clutch size promote winter survival in cold habitats, the fitness advantage her offspring receive is compounded. These benefits would be leveraged further if, as discussed above, her reproductive output were distributed among multiple nesting locations. Our finding that mature females in the CLNWR population deploy several clutches of many relatively small-bodied hatchlings supports the contention that such life-history traits have been shaped by constraints imposed by a harsh winter environment.

Acknowledgments

We thank P. Baker, J. Larson, M. French, and P. Meyer for assisting with the location and sampling of turtle nests and S. Hankison, N. Ruehl, and N. Mizel for technical assistance with the laboratory procedures. P. Baker, M. Wright, and three anonymous reviewers offered constructive comments on the manuscript, and R. Schaefer provided guidance with the statistical analyses. This work was supported by grants from the National Science Foundation (IBN 9817087) and the National Institutes of Health (NIDDKD 1 R15DK54034-01A2) to J.P.C.

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