

SOIL HYDRIC CHARACTERISTICS AND ENVIRONMENTAL ICE NUCLEI INFLUENCE SUPERCOOLING CAPACITY OF HATCHLING PAINTED TURTLES *CHRYSEMYS PICTA*

JON P. COSTANZO^{1,*}, JACQUELINE D. LITZGUS¹, JOHN B. IVERSON² AND RICHARD E. LEE, JR¹

¹Department of Zoology, Miami University, Oxford, OH 45056, USA and ²Department of Biology, Earlham College, Richmond, IN 47374, USA

*e-mail: costanjp@muohio.edu

Accepted 19 September; published on WWW 26 October 1998

Summary

Hatchling painted turtles (*Chrysemys picta*) hibernate in their shallow natal nests where temperatures occasionally fall below -10°C during cold winters. Because the thermal limit of freeze tolerance in this species is approximately -4°C , hatchlings rely on supercooling to survive exposure to extreme cold. We investigated the influence of environmental ice nuclei on susceptibility to inoculative freezing in hatchling *C. picta* indigenous to the Sandhills of west-central Nebraska. In the absence of external ice nuclei, hatchlings cooled to $-14.6 \pm 1.9^{\circ}\text{C}$ (mean \pm S.E.M.; $N=5$) before spontaneously freezing. Supercooling capacity varied markedly among turtles cooled in physical contact with sandy soil collected from nesting locales or samples of the native soil to which water-binding agents (clay or peat) had been added, despite the fact that all substrata contained the same amount of moisture (7.5 % moisture, w/w). The temperature of crystallization (T_c) of turtles exposed to frozen native soil was $-1.6 \pm 0.4^{\circ}\text{C}$ ($N=5$), whereas turtles exposed to frozen soil/clay and soil/peat mixtures supercooled extensively (mean T_c values approximately -13°C). Hatchlings cooled in contact with drier (≤ 4 % moisture) native soil also supercooled

extensively. Thus, inoculative freezing is promoted by exposure to sandy soil containing abundant moisture and little clay or organic matter. Soil collected at turtle nesting locales in mid and late winter contained variable amounts of moisture (4–15 % w/w) and organic matter (1–3 % w/w). In addition to ice, the soil at turtle nesting locales may harbor inorganic and organic ice nuclei that may also seed the freezing of hatchlings. Bulk samples of native soil, which were autoclaved to destroy any organic nuclei, nucleated aqueous solutions at approximately -7°C (T_c range -6.1 to -8.2°C). Non-autoclaved samples contained water-extractable, presumably organic, ice nuclei (T_c range -4.4 to -5.3°C). Ice nuclei of both classes varied in potency among turtle nesting locales. Interaction with ice nuclei in the winter microenvironment determines whether hatchling *C. picta* remain supercooled or freeze and may ultimately account for differential mortality in nests at a given locale and for variation in winter survival rates among populations.

Key words: painted turtle, *Chrysemys picta*, supercooling, ice nucleus, soil, freeze tolerance, survival, cold hardiness.

Introduction

Painted turtles (*Chrysemys picta*) hatch in late summer but usually remain over winter within the nest chamber, which is approximately 10 cm below the ground surface (Gibbons and Nelson, 1978). In areas where snow cover persists, nest temperatures rarely fall below freezing (Breitenbach *et al.* 1984). However, in northern regions where snow cover is sparse or transient, hatchlings are commonly exposed to temperatures below the equilibrium freezing point of their tissues (approximately -0.6°C) and occasionally to temperatures below -10°C (Woolverton, 1963; DePari, 1988; Costanzo *et al.* 1995; Packard, 1997; Packard *et al.* 1997). Most chilling episodes are of brief duration (e.g. several hours to a few days), although some persist for a week or more, and turtles may be exposed to many such events over the course of

the winter (Costanzo *et al.* 1995; Packard, 1997; Packard *et al.* 1997).

The survival of hatchling *C. picta* at subzero temperatures is promoted by their capacities for freeze tolerance and supercooling. Only relatively high temperatures (e.g. -4°C or above) may be tolerated in the frozen state (Storey *et al.* 1988; Churchill and Storey, 1992), whereas turtles may survive exposure to markedly lower temperatures by remaining supercooled (Packard and Janzen, 1996; Packard, 1997; Packard *et al.* 1997; Packard and Packard, 1997). The supercooling capacity of *C. picta* (to -20°C) is by far the best known among vertebrates and rivals that of some invertebrates (Lee and Costanzo, 1998).

Although turtles may supercool extensively under idealized

laboratory conditions (i.e. in the absence of external ice nuclei), their supercooling capacity in nature may be constrained by environmental ice nuclei that seed the freezing of supercooled tissues. In their nests, turtles may come into physical contact with ice formed by the freezing of the soil solution. They may also encounter other ice nuclei, such as inorganic crystals (Mason and Maybank, 1958; Shen *et al.* 1977), organic compounds (Power and Power, 1962; Fukuta, 1966) and microorganisms (Hirano and Upper, 1995), that commonly occur in soil. Whether such agents ultimately inoculate the body tissues depends on the nature and extent of their interaction with the animal, which in turn is governed by various physical properties of the substratum. To investigate these relationships, we conducted field and laboratory experiments to determine the roles of substratum composition and moisture content, and of ice nuclei present in native soil, in determining the susceptibility of hatchling *C. picta* to inoculative freezing.

Materials and methods

Source of animals

Eggs of *Chrysemys picta bellii* (Gray) were collected in summer 1997 near Gimlet Lake, Crescent Lake National Wildlife Refuge, Garden County, west-central Nebraska (41°N, 102°W). A description of the study site is given in our initial report on the cold hardiness of turtles indigenous to the Sandhills (Costanzo *et al.* 1995). Eggs were harvested from oxytocin-treated females (Etchberger *et al.* 1992), transported to the laboratory and incubated in moist vermiculite (1.0 g water g⁻¹ vermiculite; water potential approximately -150 kPa), at approximately 29 °C, until they hatched in late August. Hatchlings were transferred to darkened plastic boxes containing damp vermiculite (0.5 g water g⁻¹ vermiculite; water potential approximately -350 kPa) in which they were denied food and free water and kept until used. For the first month following hatching, turtles were exposed to a temperature of 20 °C in darkness to simulate conditions within the nest during late summer. Subsequently, they were cold-acclimated inside a darkened environmental chamber following a regimen inspired by field data for this population (Costanzo *et al.* 1995). Starting on 1 October 1997, turtles were sequentially exposed to 15 °C and 10 °C for 30 days at each temperature. Thereafter, they were held at 5 °C. In late winter, when they were used in experiments, hatchlings ($N=33$) weighed 3.8 ± 0.1 g and contained 3.78 ± 0.05 g water g⁻¹ dry mass (means \pm S.E.M.).

Experimental substrata

The principal substratum used in inoculation trials was collected in September 1996 at the field site, where nesting soils are light, well-drained sands or loamy sands (sand, 91 %; silt, 1 %; clay, 8 %; Costanzo *et al.* 1995). Samples of soil were collected at a depth of 10 cm from seven locales, each less than 0.5 m from a *C. picta* nest constructed the previous year. Soil was kept chilled (5 °C) in covered containers until used. The 'native soil' substratum used in turtle inoculation experiments

was a mixture of samples collected from all locales that had been sieved to remove large aggregates. Some inoculation trials were conducted using native soil augmented with clay or organic matter. These composite substrata were prepared by mixing nine parts of native soil with one part of clay (domestic cat litter, volcanic origin), which had been pulverized with a mortar and pestle, or horticultural peat that had been passed through a 2 mm² sieve. Before use, all substrata were autoclaved to destroy any organic ice nuclei, dried in an oven (65 °C) and then hydrated to the prescribed level using deionized, autoclaved water. We cooled a 50 g sample of each prepared substratum to -20 °C to determine whether its solution was freezable. Plots of temperature, constructed using single-channel temperature loggers (Onset Computer StowAway XTI; Pocasset, MA, USA), were inspected for the appearance of a freezing exotherm, the nearly instantaneous rise in temperature associated with the onset of crystallization.

Inoculation trials

Turtles were removed from their holding boxes, cleaned of adhering vermiculite using a fine-haired brush and held in sheltered open cups (5 °C, 75–80% relative humidity, in darkness) for 24 h to permit evaporation of surface moisture. After drying, the junction of a 30 gauge, copper-constantan thermocouple was glued to the carapace, and the turtle was placed vertically in a 50 ml plastic tube containing approximately 12 g of prepared substratum that was firmly tamped around the animal. More or less substratum was used in an effort to maintain a substratum:turtle mass ratio of 4:1. A pilot study showed that this protocol provided sufficient material to envelop the turtle fully and also ensured that the exotherm associated with the freezing of the turtle's tissues would be readily distinguished from that of the freezing substratum. A piece of porous plastic foam inserted into the tube insulated the space above the soil, thereby aiding detection of the turtle's exotherm. Turtles were exposed to substrata within the tubes for 24 h (5 °C, in darkness) before inoculation trials commenced.

Inoculation trials were conducted by progressively cooling turtles, which were enveloped in frozen substratum, until they themselves froze. In one experiment, we compared the temperatures of crystallization (T_c) of turtles cooled in native soil ($N=4$) with those of turtles cooled in composite substrata ($N=5$ in soil/clay mixture; $N=5$ in soil/peat mixture) hydrated to 7.5%. We also compared T_c values of turtles cooled in native soil hydrated to 7.5% with those of turtles cooled in native soil hydrated to 15% ($N=3$), 3.75% ($N=4$) and 1.9% ($N=4$). Control trials consisted of cooling additional turtles ($N=5$) in clean, dry tubes without substratum.

Inoculation trials were initiated by placing the tubes vertically into a refrigerated ethanol bath (Neslab, model RTE 140; Portsmouth, NH, USA). Although in nature soil supercools little, if at all (Hillel, 1971), the small amounts used in laboratory studies frequently do so. We ensured that substrata were frozen during inoculation trials to avoid underestimating the susceptibility of hatchlings to ice inoculation (see Packard and Janzen, 1996). Substrata were frozen by chilling them to

approximately -0.4°C , a temperature at which they (but not the turtles) could freeze, and inoculating them with minute ice crystals. These seed crystals were formed by briefly applying aerosol coolant to the exterior of the tube, causing condensate to freeze on the inner surface of the wall of the tube. This temperature was maintained for 1 h to permit the substratum to freeze thoroughly, after which the temperature inside the tube was reduced at $0.5^{\circ}\text{C h}^{-1}$ until all the turtles had produced an exotherm. During cooling, turtle temperature, as registered by the thermocouple, was logged at 30 s intervals on a chart recorder (Omega, model RD3752; Stamford, CT, USA). Turtles were subsequently weighed to an accuracy of 0.01 g and dried at 65°C to constant mass to determine the moisture content of the carcass.

Physical and hydric characteristics of experimental substrata and nesting soil

We characterized the substrata used in inoculation trials and samples of soil in which hatchling *C. picta* overwinters. The latter were collected at the field site from five locations where turtles had nested in previous years. Soil was collected from each locale at a depth of 10 cm (the depth of nest chambers) during late January and early March, placed in air-tight vessels, and shipped by express carrier to Miami University. Water content was determined from the mass lost by samples ($N=3$ replicates) by drying them at 65°C . The water-holding capacity (i.e. the water content at saturation) of these samples, and of the experimental substrata, was determined from the amount of deionized water that each ($N=3$ replicates) absorbed and held against gravity. Organic content was determined from the mass of residue remaining after incinerating the samples at 550°C for approximately 21 h ($N=3$ replicates). The water potentials of the soil samples at field water content and of the prepared experimental substrata were determined using thermocouple psychrometry ($N=4$ replicates) using a sample chamber (Wescor, model C-52; Logan, UT, USA) and dew-point microvoltmeter (Wescor, model HR-33T). The mean bulk density (the ratio of substratum mass to unit volume), particle density and porosity (an index of relative pore volume) were measured on oven-dried samples of each experimental substratum (Troeh and Palmer, 1967).

Activity of inorganic ice nuclei

We determined relative ice-nucleating activity in autoclaved samples of experimental substratum or nesting soil by comparing the T_c values of small amounts of water mixed with the material with the T_c values of equivalent quantities of water alone which, lacking potent ice nuclei, supercool in a volume-dependent manner (Vali, 1991). The water used in these trials (and all other experiments) was obtained from a reverse-osmosis ultrapurification system ($0.2\ \mu\text{m}$ filter; Dayton Water Systems, Dayton, OH, USA). A quantity ($100\ \text{mm}^3$) of air-dried substratum was placed in a 0.5 ml polypropylene microfuge tube to which $12.5\ \mu\text{l}$ of water was added. The contents were mixed by vortexing and sedimented by gentle centrifugation (180 g, 3 min). Each tube had the sensing

junction of a 36 gauge copper-constantan thermocouple taped to its exterior and was inserted into a dry 20 ml test tube. Samples ($N=12$ replicates) were chilled by submerging the test tubes in a refrigerated ethanol bath. After they had equilibrated at approximately 0°C , samples were further cooled ($1.5^{\circ}\text{C min}^{-1}$) until the water within them froze. The T_c of each sample was read from the output of a datalogger to which the thermocouples were connected. The samples were thawed, a quantity of water equal to that contained was added, and the cooling procedure was repeated. These steps were iterated until data had been collected for sample water volumes ranging from 12.5 to $100\ \mu\text{l}$. The ice-nucleating activity of bulk samples of nesting soil ($N=6$ replicates for each of the five locales) was determined in a similar manner, except that only $12.5\ \mu\text{l}$ water volumes were used. Experimental substrata and samples of nesting soil (as well as the deionized water, microfuge tubes and utensils) were autoclaved to eliminate organic ice nuclei; thus, the ice-nucleating activity expressed in these materials probably reflects the action of inorganic agents.

Activity of water-extractable ice nuclei

The ice-nucleating activity of water-extractable nuclei in soil was estimated from the mean T_c of washings, which were prepared by vortexing the soil and sterilized, deionized water ($0.5\ \text{g water g}^{-1}\ \text{soil}$) for 60 s and removing the coarse particulates by centrifugation (180 g, 3 min). The supernatant (=washing) was expressed through a $5\ \mu\text{m}$ disk filter to remove fine particulates. A $10\ \mu\text{l}$ sample of washing was drawn into the center of a $20\ \mu\text{l}$ glass microcapillary tube (such that the fluid column was bounded by equal volumes of air) whose ends were sealed with clay. The sensing junction of a 36 gauge copper-constantan thermocouple was taped to the tube, which was then inserted into a 20 ml test tube submerged in a refrigerated ethanol bath. After thermoequilibrating at approximately 0°C , samples were cooled at $1.5^{\circ}\text{C min}^{-1}$ until each produced an exotherm. We tested 12 replicates from each washing. In some cases, two washings were prepared from each sample; thus, the mean T_c representing each sample was based on 24 values. Using this protocol, we measured the activity of water-extractable ice nuclei in the native soil used in inoculation trials (both natural and autoclaved samples) and in samples of nesting soil collected in winter. The deionized water, filters, utensils and vessels used in preparing the washings were autoclaved prior to use. The potency of the ice nuclei extracted from soil samples was estimated by comparing the mean T_c of the washings with that of sterilized, deionized water. Previous study (J. P. Costanzo, J. D. Litzgus, J. B. Iverson and R. E. Lee, unpublished data) determined the sensitivity of these ice nuclei to autoclaving, which generally destroys organic nucleating agents (Vali, 1995).

Statistical inferences

Sample means were compared using the *t*-test or analysis of variance (ANOVA), with multiple comparisons determined using Bonferroni tests. Simple or multiple regressions were used to analyze relationships between various characteristics

of nesting soil. Significance of statistical analyses was accepted at $P < 0.05$. Mean values are reported \pm S.E.M. *Post-hoc* analyses showed that turtles in the various treatment groups did not differ in body mass or body water content.

Results

Effect of soil composition on inoculation susceptibility

The physical and hydric characteristics of the substrata to which turtles were exposed in inoculation trials are given in Table 1. As expected, augmenting native soil with clay or peat markedly increased its water-holding capacity and decreased its water potential. The soil/peat mixture had a relatively lower bulk density and particle density, and a higher porosity, than native soil and the soil/clay mixture. Freezing exotherms were produced by all substrata (moisture content 7.5%) used in this experiment, indicating that ice crystals were present during inoculation trials.

Control turtles, cooled in tubes without substratum (i.e. in the absence of external ice nuclei), supercooled extensively ($T_c = -14.6 \pm 1.9^\circ\text{C}$), whereas turtles cooled in contact with frozen native soil themselves froze at relatively high temperatures ($T_c = -1.6 \pm 0.4^\circ\text{C}$; Fig. 1). Adding clay or peat to the soil fully ameliorated this effect since the mean T_c values for these groups did not differ from that of control turtles cooled in tubes without substratum (Fig. 1). Thus, substratum composition markedly influenced the susceptibility of turtles to inoculative freezing ($F_{3,17} = 18.6$, $P < 0.0001$).

Effect of substratum moisture content on inoculation susceptibility

Samples of native soil hydrated to 15.0, 7.5 or 3.75% produced freezing exotherms, whereas the solution in soil hydrated to 1.9% was unfreezable. The inoculation susceptibility of turtles cooled in contact with frozen native soil

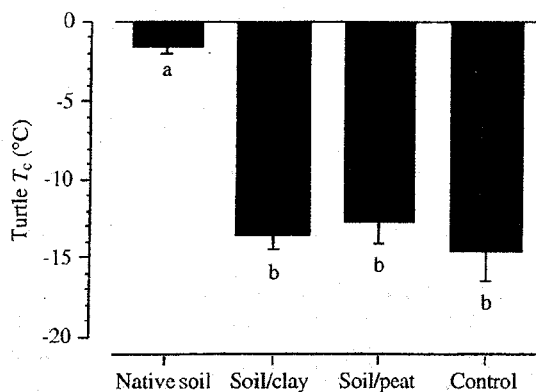


Fig. 1. Temperatures of crystallization (T_c) of hatchling *Chrysemys picta* cooled in contact with frozen native soil, or with frozen native soil augmented with 10% (w/w) clay or peat, or in tubes without substratum (control). The moisture content of substrata was 7.5%. Mean values (\pm S.E.M., $N=4-5$ replicates per group) identified by different letters are statistically different ($P < 0.05$).

Table 1. *Hydric and physical characteristics of experimental substrata used in tests of ice inoculation in hatchling painted turtles*

Variable	Native soil	Soil/clay	Soil/peat
Organic content (%)	1.7	1.9	12.4
Water potential (kPa)			
15% moisture	-105	-140	-230
7.5% moisture	-160	-400	-365
3.75% moisture	-360	ND	-9600
1.9% moisture	-3400	ND	ND
Saturation water content (%)	21.3	32.9	70.8
Bulk density (g cm^{-3})	1.5	1.5	1.0
Particle density (g cm^{-3})	2.6	2.6	1.2
Porosity (%)	50	51	59

Mixtures of autoclaved native soil and clay or peat were formulated to 90/10 (w/w).

Organic content and saturation water content are expressed as a percentage of dry mass (w/w).

ND, could not be determined because the water potential was below the limit of detection.

was strongly dependent on soil moisture content ($F_{3,14} = 23.8$, $P < 0.0001$; Fig. 2). Little supercooling occurred in turtles exposed to either of the wetter substrata (15 and 7.5%), whereas turtles tested in soil hydrated to 3.75% or 1.9% supercooled extensively. The capacity for supercooling exhibited by turtles in soil hydrated to 3.75% was similar to that in soil hydrated to 1.9%, despite the fact that the drier substratum contained no ice crystals.

Characteristics of nesting soil during winter

The moisture contents of soil sampled from five nesting locales in mid and late winter varied markedly among locales ($F_{4,24} = 285.5$, $P < 0.0001$) and between collections ($F_{1,21} = 51.9$, $P < 0.0001$). Values ranged from 4 to 15% in mid winter and from 5 to 12% in late winter (Table 2). Soil moisture content was greater in relatively protected sites where precipitation may have accumulated. The organic content of soil averaged approximately 1.5–1.7% (range 0.9–2.5%) and varied among collecting locales ($F_{4,24} = 23.2$, $P < 0.0001$). No difference in soil organic content occurred between mid winter and late winter samples ($F_{1,21} = 3.5$, $P = 0.077$). The organic content and water content were directly correlated ($r^2 = 0.70$, $F_{1,9} = 18.4$, $P = 0.003$). The water potential of the soil varied among locales ($F_{4,34} = 3.4$, $P = 0.022$) but did not differ between collections ($F_{1,31} = 1.5$, $P = 0.23$). Water potential was not correlated ($r^2 = 0.24$, $F_{3,9} = 0.62$, $P = 0.63$) with either moisture content or organic content.

Activity of inorganic ice nuclei in experimental substrata and nesting soil

Ice-nucleating activity reflecting the action of non-organic ice nuclei was expressed in autoclaved, bulk samples of native soil, soil/clay mixture and soil/peat mixture (Fig. 3). The relationship in which the T_c of a solution lacking potent ice nuclei depends on the volume of the solution (Vali, 1991) was

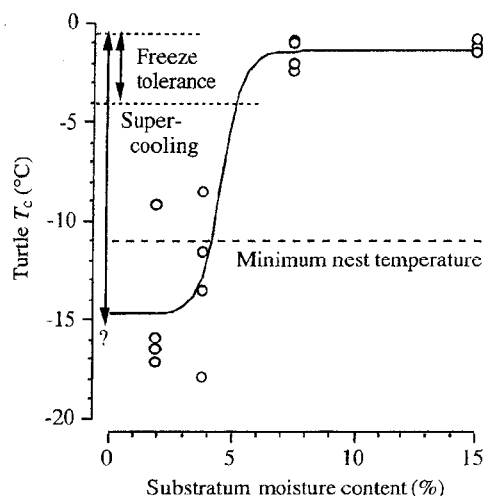


Fig. 2. Temperatures of crystallization (T_c) of individual hatchling *Chrysemys picta* cooled in contact with frozen native soil as a function of soil moisture content. Double-headed arrows show the thermal ranges of freeze tolerance (Storey *et al.* 1988; Churchill and Storey, 1992; Costanzo *et al.* 1995) and survival in the supercooled state (Packard *et al.* 1997; J. P. Costanzo, J. D. Litzgus, J. B. Iverson, R. E. Lee, unpublished data) for hatchling *C. picta*. The typical minimum temperature occurring in *C. picta* nests during cold winters is also indicated (Woolverton, 1963; DePari, 1988; Costanzo *et al.* 1995; Packard, 1997). The curve was fitted to data points using nonlinear regression ($r^2=0.87$, d.f.=12, absolute sum of squares=89.7) and is described by the logistic equation: $y=-14.7+[13.3/(1+10^{(4.5-x)})]$.

demonstrated for samples of sterilized, deionized water ($F_{3,47}=11.7$, $P<0.0001$); however, this relationship was

supplanted by the activity of ice nuclei in samples containing native soil ($F_{3,47}=0.1$, $P=0.95$) or the soil/peat mixture ($F_{3,47}=2.0$, $P=0.12$). A marginally significant ($F_{3,47}=3.0$, $P=0.04$) volume-dependence was found for the soil/clay mixture, although these samples also exhibited ice-nucleating activity over a relatively narrow range of temperatures (-6.1 to -7.0 °C). Addition of clay or peat to native soil tended to depress its ice-nucleating activity (Fig. 3).

As was the case with the native soil used in inoculation trials, soil collected from nesting locales inoculated a 12.5 μ l volume of water at approximately -7 °C (Table 2). Ice-nucleating activity in these autoclaved samples varied among the five locales ($F_{4,54}=40.4$, $P<0.0001$; range -6.1 to -8.2 °C), but was similar between mid winter and late winter samples ($F_{1,51}=2.4$, $P=0.13$).

Activity of water-extractable ice nuclei in experimental substrata and nesting soil

Ice-nucleating activity in washings prepared from autoclaved samples of the native soil used in inoculation trials ($T_c=-13.7\pm 0.7$ °C; $N=12$) and in sterilized, deionized water ($T_c=-20.3\pm 0.7$ °C; $N=12$) was significantly lower ($F_{2,35}=176.9$, $P<0.0001$) than that in washings prepared from non-autoclaved samples of native soil ($T_c=-4.8\pm 0.09$ °C; $N=12$). This result attests to the efficacy of autoclaving in eliminating potent, water-extractable (organic?) ice nuclei.

The activity of water-extractable ice nuclei in nesting soil varied among collection locales ($F_{4,232}=47.7$, $P<0.0001$; range -4.4 to -5.3 °C; Table 2). Activity was marginally lower in late winter than in mid winter ($F_{1,229}=4.2$, $P=0.042$). There was no correlation between the activity of water-extractable ice nuclei and the activity of nuclei expressed in corresponding bulk

Table 2. Hydric characteristics and activity of ice nuclei in nesting soil during winter

Variable	Collection locale					Mean
	1	2	3	4	5	
Mid winter						
Moisture content (%)	9.0	14.6	12.0	9.1	4.4	9.8 \pm 0.9
Organic content (%)	1.3	2.5	1.7	1.3	1.0	1.7 \pm 0.2
Water potential (kPa)	-100	-80	-110	-85	-60	-90 \pm 9
Ice-nucleating activity (°C)						
Autoclaved bulk soil	-7.7	-7.8	-6.2	-7.7	-7.9	-7.4 \pm 0.1
Soil washing	-4.8	-4.9	-4.4	-4.6	-4.5	-4.6 \pm 0.03
Late winter						
Moisture content (%)	5.3	12.0	9.9	10.7	4.8	8.5 \pm 0.8
Organic content (%)	1.5	2.5	1.6	1.3	0.9	1.5 \pm 0.1
Water potential (kPa)	-110	-70	-80	-45	-90	-80 \pm 6
Ice-nucleating activity (°C)						
Autoclaved, bulk soil	-8.2	-7.3	-6.1	-6.8	-8.0	-7.3 \pm 0.2
Soil washing	-5.3	-4.7	-4.4	-4.7	-4.4	-4.7 \pm 0.04

Mean values \pm S.E.M. are based on data for soil, collected at a depth of 10 cm, from five collection locales within each row.

Moisture content and organic content are expressed as a percentage of dry mass.

The number of replicates (N) in each determination is as follows: moisture content (3); organic content (3); water potential (4); ice-nucleating activity, autoclaved bulk soil (6), soil washing (23–24).

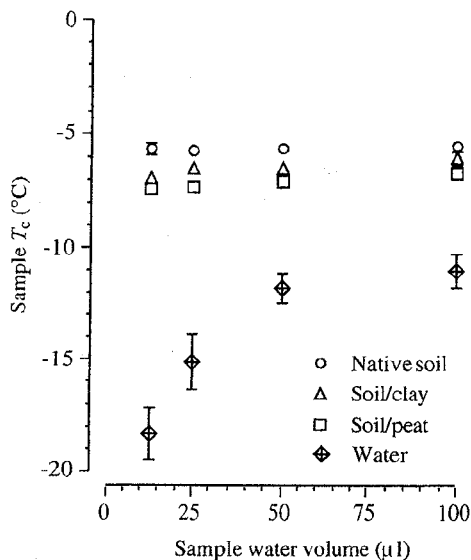


Fig. 3. Ice-nucleating activity, as measured by the temperature of crystallization (T_c) of samples of experimental substrata used in tests of inoculative freezing of hatchling *Chrysemys picta* relative to deionized water. Samples contained only water or water with 100 mm³ of autoclaved substratum. Values are means \pm S.E.M., $N=12$ replicates per group. Most error bars are smaller than the symbols.

samples of autoclaved soil ($r^2=0.28$, $F_{1,9}=3.2$, $P=0.11$), suggesting that the two classes of ice nuclei are unrelated.

Discussion

Small ectothermic animals often exhibit high capacities for supercooling, in part because the probability of spontaneous ice nucleation is relatively low in small aqueous volumes (Vali, 1991; Costanzo and Lee, 1995). When isolated from external ice nuclei, hatchling *C. picta* exhibit a remarkable capacity to supercool that is much greater than can be explained by body size alone (Lee and Costanzo, 1998). Under idealized conditions, hatchlings remained supercooled at temperatures as low -17.9°C in the present study (Fig. 2) and as low as -20°C in a previous investigation (J. P. Costanzo, J. D. Litzgus, J. B. Iverson and R. E. Lee, unpublished data). Although the physiological basis for their exceptional supercooling capacity is unknown, the innate ability to remain unfrozen at such low temperatures has adaptive value for this species, which occasionally encounters temperatures below -10°C while hibernating within the natal nest (Woolverton, 1963; DePari, 1988; Costanzo *et al.* 1995; Packard, 1997; Packard *et al.* 1997). Nevertheless, under natural conditions, hatchlings may encounter ice nuclei that may seed the freezing of their body tissues. Freezing can be survived only if their tissues are inoculated at high temperatures, which permits ice to propagate slowly throughout the body, and if body temperature remains at -4°C or above; freezing at lower temperatures is lethal (Storey *et al.* 1988; Churchill and Storey, 1992; Costanzo *et al.* 1995).

Environmental factors that influence inoculative freezing are thus of paramount importance to the cold hardness of this species.

Environmental factors influencing susceptibility to inoculative freezing

The mechanical events whereby environmental ice nuclei trigger the freezing of body tissues are incompletely understood. In some insects, ice apparently infiltrates the body through respiratory pores and joints in the exoskeleton (Salt, 1963; Lee *et al.* 1996). Amphibians are highly susceptible to ice inoculation owing to the exceptional permeability of their skin (Layne *et al.* 1990; Layne, 1991). The integument of reptiles and fishes resists ice transfer better, perhaps as a result of differences in the structure and composition of the epidermis (Valerio *et al.* 1992; Costanzo and Lee, 1995, 1996). In addition, antifreeze proteins enable some fish to resist ice inoculation (DeVries, 1982), but these agents have not been found in reptiles. Regardless of species, one fundamental requirement for inoculative freezing to occur is that ice nuclei come into physical contact with the animal. The characteristics of the substratum, particularly those influencing the quantity of ice nuclei and their degree of intimacy with the animal, may be important determinants of inoculative freezing.

The inoculation susceptibility of some burrowing animals depends on the moisture content, water potential, texture and porosity of the substratum and other factors that directly or indirectly influence the abundance and distribution of ice within the substratum matrix (Salt, 1963; Layne *et al.* 1990; Lundheim and Zachariassen, 1993; Costanzo *et al.* 1995, 1997). Some ectotherms avoid ice inoculation better if the frozen substrata to which they are exposed contain clay or organic matter. By adsorbing water, these materials may attenuate and/or reduce the formation of ice within soil pores (Forge and MacGuidwin, 1992; Costanzo *et al.* 1997). The inoculation resistance of our hatchling *C. picta* cooled in contact with sandy soil was markedly improved by adding either clay or organic matter (peat) to the substratum. Because soils at our study site contain little clay or organic matter, the turtles there may freeze more often than turtles inhabiting areas with different soil types, other factors being equal. Selecting a clay-type soil for the nesting substratum not only improves the viability of the offspring during embryological development (Ratterman and Ackerman, 1989) but may also promote supercooling of hatchlings hibernating within the nest.

A recent laboratory study by Packard and Packard (1997) showed that soil composition made little difference to inoculation rates of hatchling *C. picta* cooled to -4°C , although more animals froze when cooled to -7°C in 'loamy sand' (80%) than in 'clayey soil' (33%). The effect of substratum type in the trials at -7°C might have been even more pronounced had the loamy sand not contained large amounts of organic material which, as demonstrated in the present report (Fig. 1), inhibits inoculative freezing. Notably, these authors obtained distinct results even though the loamy sand and clayey soil had similar hydric characteristics

(moisture contents, 16 and 20 %, respectively; water potential, approximately -75 kPa in both substrata).

Although the hypothesis that soil composition influences the inoculation susceptibility, and hence the cold hardiness, of hatchling *C. picta* has not been directly tested in nature, some field data shed light on this issue. Our analysis of the results presented by Packard (1997) for a Nebraskan population of *C. picta* suggests that the winter survival of hatchlings occupying nests constructed in 'loamy sand' (94 %) was significantly higher (Fisher's exact test, $P < 0.0001$) than that of turtles hibernating in 'fine sand' (65 %). From the results of a similar study conducted the following year (Packard *et al.* 1997), we note that survival was again lower for hatchlings overwintering in fine sand than in loamy sand (36 % versus 90 %, respectively; Fisher's exact test, $P < 0.0001$). Although the relative importance of soil type to survivorship in these studies is confounded by reportedly wide variation in nest temperatures, these field observations, together with our laboratory data (Fig. 1), suggest that winter survival rates of hatchling *C. picta* overwintering within nests may be linked to soil edaphics on both local and regional scales.

Effect of soil moisture on inoculation susceptibility

Our data indicate that the inoculation susceptibility of hatchling *C. picta* is strongly influenced by the moisture content of the substratum. Turtles were highly susceptible to inoculation in native soil containing more than 4 % moisture; extensive supercooling was possible only in relatively dry soil (Fig. 2). Inoculation was avoided by most turtles cooled in these drier substrata, perhaps because ice crystals were either too finely dispersed (3.75 % moisture content) or lacking altogether (1.9 % moisture). Apparently these animals spontaneously froze when their tissues reached the limit of supercooling.

The soil moisture levels at our field site varied spatially (Table 2) suggesting that, at any given time, turtles in different nests may be frozen or supercooled. All other factors being equal, local variation in soil moisture content may account for the observed differential survival of hatchlings overwintering in neighboring nests (see Packard, 1997; Packard *et al.* 1997). Soil moisture in the Sandhills may also change seasonally, with attendant implications for turtle cold hardiness. Mean soil water content ranged from 2 to 7 % in autumn, depending upon the recent history of precipitation (Costanzo *et al.* 1995), and was relatively higher (9–10 %) during mid and late winter in the present study (Table 2), which was conducted during a relatively dry period. This apparent seasonal pattern is deleterious because the soil is wettest (and the chance of ice inoculation is highest) when nest temperatures are generally at their minima (Costanzo *et al.* 1995; Packard, 1997; Packard *et al.* 1997).

Our laboratory and field data support the hypothesis that the cold hardiness strategy of hatchling *C. picta* encompasses both freeze tolerance and supercooling capacities. According to this view, survival is promoted by freeze tolerance when environmental and physiological conditions facilitate inoculation at relatively high temperatures and frozen turtles remain at temperatures at or above -4 °C, whereas supercooling

is the survival strategy whenever inoculative freezing can be avoided (Costanzo *et al.* 1995). The logistic relationship between the inoculation susceptibility of hatchlings and soil moisture content promotes reliance on either freeze tolerance or supercooling for surviving a given chilling episode (Fig. 2). Hatchlings exposed to relatively damp soil (i.e. water content ≥ 7.5 %) are readily inoculated at body temperatures conducive to freezing survival, whereas turtles in slightly drier soil (i.e. water content ≤ 4 %) may avoid ice inoculation, remaining supercooled over the range of temperatures normally encountered in nests during winter. Given the narrow range of soil moisture levels (4–5 %; Fig. 2) that cause turtles to freeze at lethal temperatures, perhaps relatively few turtles suffer this fate. We caution that our study of these relationships used soil that was autoclaved to eliminate potentially confounding effects of water-extractable ice nuclei. Thus, although the general pattern of response may accurately reflect that of turtles overwintering in natural nests, they may be more susceptible to inoculative freezing than our data suggest.

Ice nuclei in the winter microenvironment

The roles of environmental ice nuclei other than ice crystals in the cold hardiness of ectothermic animals are poorly understood (Lee and Costanzo, 1998). Many ice nuclei commonly occurring in soil, vegetative detritus and the atmosphere are complexes of inorganic and organic elements formed during the decay of organic materials (Vali, 1991). The soil in which *C. picta* nests may contain a host of inorganic particulates (Mason and Maybank, 1958; Shen *et al.* 1977), organic compounds (Power and Power, 1962; Fukuta, 1966) and microorganisms (Hirano and Upper, 1995). Our initial investigation identified two classes of agents present in soil in which hatchling *C. picta* overwinters (J. P. Costanzo, J. D. Litzgus, J. B. Iverson, R. E. Lee, unpublished data). One class was represented by ice nuclei that were active in bulk samples of autoclaved soil; the other was represented by extremely small (<10 nm diameter) nuclei that were water-extractable and whose activity was largely destroyed by autoclaving. Limited evidence suggests that the autoclave-resistant ice nuclei active in bulk soil samples were inorganic particulates, whereas the autoclave-sensitive ones were organic and were possibly associated with ice-nucleating microorganisms. Both types of ice nuclei inoculated hatchling *C. picta*, even in the absence of environmental moisture. When they were cooled in dry, autoclaved nest soil, supercooling capacity was reduced in 27 % of the turtles, which froze at approximately -10 °C, 8 °C higher than the others. In the present study, only one of the four hatchlings tested in unfreezeable native soil (1.9 % moisture group; Fig. 2) was apparently inoculated, at -9.2 °C, by chance contact with particulate ice nuclei. Although these ice nuclei, which are relatively inefficient at inoculating tissues, may affect relatively few turtles, their effect is profound because turtles cannot survive freezing at the T_c values that result.

Interaction with ice nuclei in the winter environment may have important implications for the cold hardiness of hatchling

C. picta. The physical and hydric attributes of the soil governing the amount and distribution of ice in the substratum, and thus the intimacy between ice crystals and the animal, may promote either inoculation at the high temperatures conducive to freezing survival or avoidance of ice inoculation and extensive supercooling. Whether survival of a given chilling episode is attributable to freeze tolerance or to supercooling, or whether hatchlings die from freezing at critically low temperatures, ultimately depends on a complex interplay among soil characteristics, ice nuclei and nest temperatures.

We gratefully acknowledge technical assistance by M. French, C. Kostizen and C. Wentzel. We also thank M. Wright and J. Layne for criticizing the manuscript. This work was supported, in part, by grants from the National Science Foundation (IBN 9507437) and National Institutes of Health (1 R15 DK48067-01A1) to J.P.C.

References

- BREITENBACH, G. L., CONGDON, J. D. AND VAN LOBEN SELS, R. C. (1984). Winter temperatures of *Chrysemys picta* nests in Michigan: effects on hatchling survival. *Herpetologica* **40**, 76–81.
- CHURCHILL, T. A. AND STOREY, K. B. (1992). Natural freezing survival by painted turtles *Chrysemys picta marginata* and *C. picta bellii*. *Am. J. Physiol.* **262**, R530–R537.
- COSTANZO, J. P., IVERSON, J. B., WRIGHT, M. F. AND LEE, R. E. (1995). Cold hardiness and overwintering strategies of hatchlings in an assemblage of northern turtles. *Ecology* **76**, 1772–1785.
- COSTANZO, J. P. AND LEE, R. E. (1994). Biophysical and physiological responses promoting freeze tolerance in vertebrates. *News physiol. Sci.* **9**, 252–256.
- COSTANZO, J. P. AND LEE, R. E. (1995). Supercooling and ice nucleation in vertebrates. In *Biological Ice Nucleation and its Applications* (ed. R. E. Lee, Jr, G. J. Warren and L. V. Gusta), pp. 221–237. St Paul, MN: American Phytopathological Society Press.
- COSTANZO, J. P. AND LEE, R. E. (1996). Mini review: ice nucleation in freeze-tolerant vertebrates. *Cryo-Lett.* **17**, 111–118.
- COSTANZO, J. P., MOORE, J. B., LEE, R. E., KAUFMAN, P. E. AND WYMAN, J. A. (1997). Influence of soil hydric parameters on the winter cold hardiness of a burrowing beetle, *Leptinotarsa decemlineata* (Say). *J. comp. Physiol.* **167**, 169–176.
- DEPARI, J. A. (1988). Overwintering in the nest chamber by hatchling painted turtles, *Chrysemys picta*, in northern New Jersey. PhD dissertation, Rutgers University, Rutgers, NJ, USA.
- DEVRIES, A. L. (1982). Biological antifreezes in coldwater fishes. *Comp. Biochem. Physiol.* **73A**, 627–640.
- ETCHBERGER, C., EWERT, M. A., RAPER, B. A. AND NELSON, C. E. (1992). Do low incubation temperatures yield females in painted turtles? *Can. J. Zool.* **70**, 391–394.
- FORGE, T. A. AND MACGUIDWIN, A. E. (1992). Effects of water potential and temperature on survival of the nematode *Meloidogyne hapla*. *Can. J. Zool.* **70**, 1553–1560.
- FUKUTA, N. (1966). Experimental studies of organic ice nuclei. *J. atmos. Sci.* **23**, 191–196.
- GIBBONS, J. W. AND NELSON, D. H. (1978). The evolutionary significance of delayed emergence from the nest by hatchling turtles. *Evolution* **32**, 297–303.
- HILLEL, D. (1971). *Soil and Water: Physical Principles and Processes*. New York: Academic Press.
- HIRANO, S. S. AND UPPER, C. D. (1995). Ecology of ice nucleation-active bacteria. In *Biological Ice Nucleation and its Applications* (ed. R. E. Lee, Jr, G. J. Warren and L. V. Gusta), pp. 41–61. St Paul, MN: American Phytopathological Society Press.
- LAYNE, J. R., JR (1991). External ice triggers freezing in freeze-tolerant frogs at temperatures above their supercooling point. *J. Herpetol.* **25**, 129–130.
- LAYNE, J. R., JR, LEE, R. E. AND HUANG, J. L. (1990). Inoculation triggers freezing at high subzero temperatures in a freeze-tolerant frog (*Rana sylvatica*) and insect (*Eurosta solidaginis*). *Can. J. Zool.* **68**, 506–510.
- LEE, R. E. AND COSTANZO, J. P. (1998). Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *A. Rev. Physiol.* **60**, 55–72.
- LEE, R. E., STEIGERWALD, K. A., WYMAN, J. A., COSTANZO, J. P. AND LEE, M. R. (1996). Anatomic site of application of ice-nucleating active bacteria affects supercooling in the Colorado Potato beetle (Coleoptera: Chrysomelidae). *Env. Ent.* **25**, 465–469.
- LUNDHEIM, R. AND ZACHARIASSEN, K. E. (1993). Water balance of overwintering beetles in relation to strategies for cold tolerance. *J. comp. Physiol.* **163**, 1–4.
- MASON, B. J. AND MAYBANK, J. (1958). Ice-nucleating properties of some natural mineral dusts. *Qt. J. R. meteor. Soc.* **84**, 235–241.
- PACKARD, G. C. (1997). Temperatures during winter in nests with hatchling painted turtles (*Chrysemys picta*). *Herpetologica* **53**, 89–95.
- PACKARD, G. C., FASANO, S. L., ATTAWAY, M. B., LOHMILLER, L. D. AND LYNCH, T. L. (1997). Thermal environment for overwintering hatchlings of the painted turtle (*Chrysemys picta*). *Can. J. Zool.* **75**, 401–406.
- PACKARD, G. C. AND JANZEN, F. J. (1996). Interpopulational variation in the cold-tolerance of hatchling painted turtles. *J. therm. Biol.* **21**, 183–190.
- PACKARD, G. C. AND PACKARD, M. J. (1997). Type of soil affects survival by overwintering hatchlings of the painted turtle. *J. therm. Biol.* **22**, 53–58.
- POWER, B. A. AND POWER, R. F. (1962). Some amino-acids as ice nucleators. *Nature* **194**, 1170–1171.
- RATTERMAN, R. J. AND ACKERMAN, R. A. (1989). The water exchange and hydric microclimate of painted turtle (*Chrysemys picta*) eggs incubating in field nests. *Physiol. Zool.* **62**, 1059–1079.
- SALT, R. W. (1963). Delayed inoculative freezing of insects. *Can. Ent.* **95**, 1190–1202.
- SHEN, J. H., KLIER, K. AND ZETTMLOYER, A. C. (1977). Ice nucleation by micas. *J. atmos. Sci.* **34**, 957–960.
- STOREY, K. B., STOREY, J. M., BROOKS, S. P. J., CHURCHILL, T. A. AND BROOKS, R. J. (1988). Hatchling turtles survive freezing during winter hibernation. *Proc. nam. Acad. Sci. U.S.A.* **85**, 8350–8354.
- TROEH, F. R. AND PALMER, R. G. (1967). *Introductory Soil Science Laboratory Manual*. Ames, IA: Iowa State University Press.
- VALERIO, P. F., KAO, M. H. AND FLETCHER, G. L. (1992). Fish skin: an effective barrier to ice crystal propagation. *J. exp. Biol.* **164**, 135–151.
- VALI, G. (1991). Nucleation of ice. In *Atmospheric Particles and Nuclei* (ed. G. Gotz, E. Meszaros and G. Vali), pp. 131–192. Budapest: Akademiai Kiado.
- VALI, G. (1995). Principles of ice nucleation. In *Biological Ice Nucleation and its Applications* (ed. R. E. Lee, Jr, G. J. Warren and L. V. Gusta), pp. 1–28. St Paul, MN: American Phytopathological Society Press.
- WOOLVERTON, E. (1963). Winter survival of hatchling painted turtles in northern Minnesota. *Copeia* **1963**, 569–570.